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Development of a Binary Positive and Negative Protein Fragment Complementation Assay using Yeast Cytosine Deaminase

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Ce mémoire intitulé:

Development of a Binary Positive and Negative
Protein Fragment Complementation Assay using Yeast Cytosine Deaminase

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RESUME

Les essais de sélection facilitent les études en génétique, en biologie moléculaire et en biologie cellulaire. Notre laboratoire a développé plusieurs essais de complémentation de fragments protéiques (PCAs) basés sur des protéines rapportrices pour la survie, la fluorescence, les changements colorimétriques et la luminescence. Le PCA consiste à séparer le gène de la protéine rapportrice en deux et de les fusionner à deux gènes codant pour des protéines d'intérêt. Les gènes de fusion sont exprimés dans une cellule hôte et lorsque les deux protéines d'intérêt intéragissent ensemble, les fragments de la protéine rapportrice se rapprochent et se replient afin de retrouver sa conformation native de la protéine rapportrice. Les PCAs nous ont permis d'étudier les interactions protéine-protéine lors d'une pertubation dans une voie métabolique, de cribler des librairies de gènes pour identifier nouvelles interactions entre les protéines, et d'évoluer deux protéines afin d'obtenir une meilleure interaction réciproque.

Le présent travail consistait à développer un nouvel essai de complémentation de fragments protéiques avec la cytosine déaminase de *Saccharomyces cerevisiae* (yCD) pour une sélection binaire positive et négative. La yCD, une enzyme impliquée dans la voie de recyclage des pyrimidines, a été choisie car elle peut être utilisée comme protéine rapportrice pour le développement d'un essai de survie cellulaire ou de mort cellulaire avec du 5-fluorocytosine (5-FC). Le 5-FC est converti en 5-fluorouracil, un composé toxique pour les cellules, par la yCD. Pour le développement du PCA yCD, nous avons fragmenté la yCD en deux, à sept positions différentes, et fusionné chaque fragment à la fermeture éclair à leucines de GCN4 (Zip). Nous avons utilisé le PCA yCD et identifié deux sites de coupure permettant la régénération de l'activité cytosine déaminase. D'autre part, nous avons criblé les sept variants de fragments 1 [F1] contre les sept variants de fragments 2 [F2] et avons identifié une reconstitution optimale de l'activité avec la combinaison Zip-[F1]yCD cut4 et Zip-[F2]yCD cut1. Cette combinaison contient une répétition d'un fragment de la yCD localisée au niveau de la deuxième hélice α. Pour démontrer que ce PCA peut être utilisé chez les cellules de mammifères, nous avons placé le PCA yCD sous le contrôle des promoteurs des gènes de l'antigène carcino-embryonnaire (CEA) et de la telomerase transcriptase inverse humaine (hTERT). Nous avons établi des lignées stables de cellules HEK 293 avec ces constructions. Les lignées stables ont été testées avec 5-FC et nous avons observé une diminution de croissance cellulaire due aux effets du PCA yCD

et du 5-FC. Une optimisation du PCA yCD sera requise pour son utilisation dans les cellules de mammifères.

MOTS CLÉs :

Selection positive et negative, essai de complémentation de fragments protéiques (PCA), essai de survie, essai de mort, cytosine déaminase de *Saccharomyces cerevisiae* (yCD), recyclage des pyrimidines, 5-fluorocytosine (5-FC), 5-fluorouracil (5-FU).

ABSTRACT:

Selection assays are fundamental for molecular biology and cell biology. Our laboratory has developed various protein-fragment complementation assays (PCAs) based on reporter proteins that when reconstituted provide for survival, are fluorescent, or can convert substrates to fluorescent, colored or luminescent products. PCA consists of rationally dissecting a reporter gene into two fragments and fusing these separated fragments to two genes of interest. The fusion genes are expressed in host cells and when the two proteins of interest interact with each other, the two fragments are brought into proximity and refold to generate the reporter protein. PCAs have been used to study protein-protein interactions, screen for unknown protein interacting partners, and engineer optimal binding protein partners.

In this work, we developed a novel Binary Positive and Negative selection PCA, which can increase the specificity of a selection system and be used for multiple applications. This novel PCA utilizes the *Saccharomyces cerevisiae* cytosine deaminase (yCD), an enzyme involved in the pyrimidine salvage pathway, which allows selection for cell survival, or cell death in the presence of 5-fluorocytosine (5-FC). 5-FC is a relatively non-toxic prodrug that can be converted to a cytotoxic compound, 5-fluorouracil by yCD. For the development of the yCD PCA, we fragmented yCD at seven different cut sites and fused each fragment to the GCN4 leucine zipper (Zip) domain, which can form a homodimer and bring the yCD fragments into close proximity. We tested the efficiency of yCD PCA using the cell death assay and demonstrated yCD PCA activity for two cut sites. In addition, we screened for yCD PCA activity by shuffling the seven variants of yCD fragment 1 [F1] against the seven variants of fragment 2 [F2]. We found an increased yCD PCA activity for the Zip-[F1]yCD cut4 and Zip-[F2]yCD cut1, which contains two fragments that overlap in the region of the α2 helix of yCD. The presence of these overlapping α2 helices in the yCD PCA could contribute to the stabilization of the full-length enzyme in a monomeric form resulting in an enhanced yCD PCA activity. We developed and demonstrated both positive and negative selection assays in yeast using a yCD PCA containing the overlapping α2 helix fragment combination that gave the best activity. We then demonstrated a binary yCD PCA designed to specifically kill a

population of mammalian cells. In this assay the two complementary Zip-fragment fusions were expressed under the control of two promoters demonstrated to be highly active in the cell line of interest. Specifically, we cloned the Zip-[F1]yCD cut4 and Zip-[F2]yCD cut1 recombinant genes under the control of the promoters of carcinoembryonic antigen (CEA) and human telomerase reverse transcriptase (hTERT) respectively, and generated stable HEK 293 cell lines containing these two constructs. Using the yCD PCA and 5-FC death selection assay, we demonstrated inhibition of cell growth of the resulting stable cell lines compared to control cell lines. Improvements in this death assay will be possible through ongoing directed evolution efforts and optimization of selection conditions. Further a survival assay for mammalian cells will be developed. We envisage many applications of both the yeast and mammalian yCD PCAs including protein interaction dissection and optimization, small molecule inhibitor screening, lineage switching in development and cancer gene therapy efforts.

KEY WORDS:

Positive negative selection, survival assay, death assay, protein-fragment complementation assay (PCA), protein-protein interaction, yeast cytosine deaminase (yCD), pyrimidine salvage pathway, 5-fluorocytosine (5-FC), 5-fluorouracil (5-FU).

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CHAPTER 1: INTRODUCTION

1.1 Selection Assays

Development of selection assays has facilitated studies in genetics, molecular biology and biochemistry. The first molecular biology selection assay was adapted from a naturally occurring resistance vector carrying a penicillinase gene, which allowed *Staphylococcus aureus* to grow in media containing erythromycin (Lindbergi, Novick R, 1973). In 1977, Bolivar *et al.* developed other cloning vectors, which were more stable and have other antibiotic resistance markers for survival selection assays. Nowadays, in addition to survival selection assays established by antibiotic resistance markers, other positive selection assays have been developed based on auxotrophic markers (Barnes 1979; Chevallier, Bloch et al. 1980; Grimm, Kohli et al. 1988). Negative selection systems have also been developed based on strategies that cause cell growth inhibition (Grimm, Kohli et al. 1988; Yagi, Nada et al. 1993). Consequently, applications of selection assays have shifted from simple phenotypic markers to sophisticated systems, which allow for simultaneous selection of multiple characteristics as discussed in the following two examples.

In some cases, combinations of multiple reporter proteins are required in order to establish a complex selection strategy. This is seen in the strategy used for generating targeted gene replacement (Capecchi 1994). The strategy consists of using two selection markers [the neomycin resistance gene (neo^r) and the Herpes Simplex Virus type I thymidine kinase gene (HSV1-TK)] as positive and negative selection reporter proteins. First, cells are selected for integrating the neo^r gene into their genome by a positive selection assay using the antibiotic neomycin (G418), which kills cells that do not carry the neo^r gene. Second, cells that integrated the disruption cassette with HSV1-TK gene at random integration sites are killed using a prodrug, ganciclovir. After the dual selection events, surviving cells represent those that harbored a targeted gene replacement.

In other cases, a single reporter protein can be used for multiple selection assays. For example, orotidine-5'-monophosphate decarboxylase (OMPdecase), a reporter protein encoded by the *URA3* gene, can be used in either a positive or a negative selection assay. The positive selection can be established in a *URA3* deletion (*ura3*$^-$) strain of *Saccharomyces cerevisiae* (S. *cerevisiae*), a uracil auxotroph, by complementing for the

gene deletion with a plasmid carrying the *URA3* gene as a selection marker for growth in minimal medium lacking uracil (Gerbaud, Fournier et al. 1979). In contrast, the negative selection assay consists of killing yeast cells carrying the *URA3* gene with 5-fluoroorotic acid (5-FOA). The combination of *URA3* gene and 5-FOA have been widely used in plasmid shuffling assays and specific targeted gene replacement (Boeke, Trueheart et al. 1987).

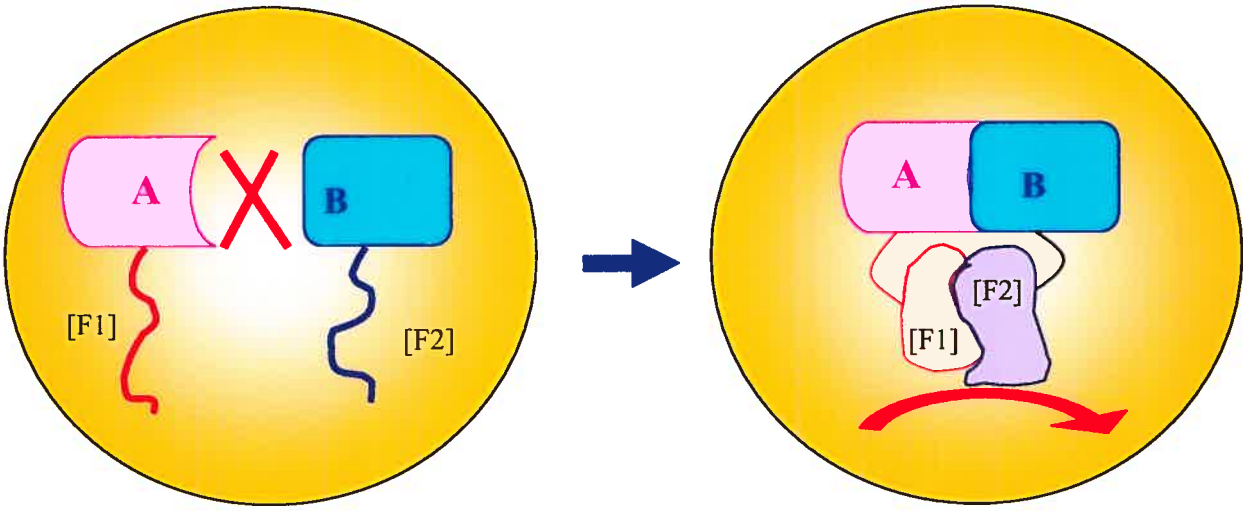
Development of multiple selection systems is both laborious and time consuming. For this reason, combining multiple selection strategies using an individual selection marker protein could increase the efficiency and provide for multiple applications from one selection system. This idea inspired us to develop a Binary Positive and Negative Selection strategy using Protein fragment Complementation Assay (PCA). As described below, PCA involves reconstitution of a protein, often an enzyme, from complementary fragments and this reconstitution is driven by the interaction of proteins to which the individual fragments are fused (Figure 1). Thus, for example, it is possible to imagine a selection system for creating stable cell lines for two genes simultaneously in which plasmids harboring the genes also contain one of two individual PCA fragment-interacting protein fusions. Expression of both PCA partners in cells that have incorporated the two plasmids would then result in reconstitution of the PCA reporter protein activity and signaling incorporation of the two genes of interest. Equally, each of the PCA pairs could be expressed under the control of two different promoters that are uniquely active in a specific cell type, therefore allowing binary lineage selection. Finally, if the PCA reporter protein could allow for positive survival selection under one set of conditions but negative, death selection under another set, one would have a binary and switchable PCA (Figure 2). A binary positive and negative PCA could increase specificity for selecting a clonal population of cells or increase specificity for killing a particular population of cells. In addition, a binary positive and negative PCA could be used to screen for molecular characteristics of interacting protein partners such as optimal binding or identification of binding motifs.

In the following sections, I will describe: a) why PCA is a good method for the establishment of this Binary Positive and Negative selection strategy; b) some specific applications of the Binary Positive and Negative PCA; c) selection of an appropriate

Figure 1. Protein fragment Complementation Assay (PCA). Fragment 1 [F1] of the reporter enzyme is fused to protein A and fragment 2 [F2] of the reporter protein is fused to protein B. When protein A and protein B interact with each other, [F1] and [F2] are brought together and fold to reconstitute the reporter protein. A biological signal will be detected in the form of cell survival, fluorescence, luminescence, etc. depending on the nature of the assay.

No interaction between A and B

A and B interact with each other

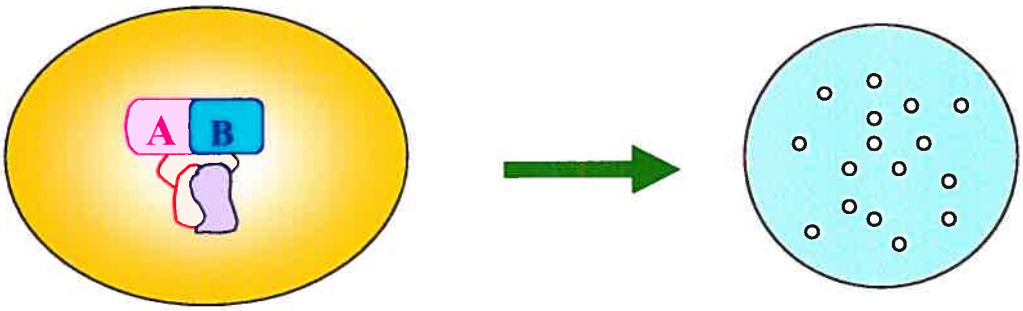


No biological signal

Biological signal: Survival
Fluorescence
Luminescence

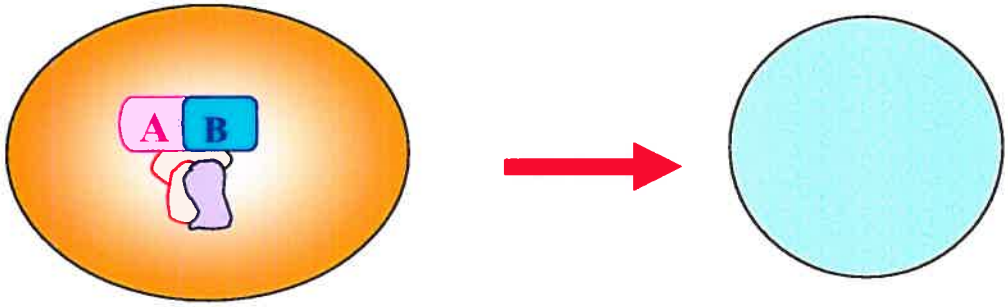
Figure 2. Binary Positive and Negative PCA. When protein A and protein B interact with each other, the reporter fragments [F1] and [F2] are brought together, fold to reconstitute the reporter protein and display an outcome of the selection assay as: A) Positive Selection PCA, where cell growth is observed or B) Negative Selection PCA, where cell death is observed.

A) Positive Selection PCA



growth colonies

B) Negative Selection PCA



death no colonies

reporter protein that can both regulate a metabolic pathway required for cell survival or inhibit cell growth under different conditions; d) determination of a cellular system for the selection assay; and e) approaches used for the development of the Binary Positive and Negative PCA.

1.2 Protein fragment Complementation Assays

Our laboratory has developed several Protein fragment Complementation Assays (PCAs) for studying dynamic protein-protein interactions in the context of living cells. PCA consists of rationally dissecting a reporter protein into two fragments and generate recombinant proteins with these separated fragments and two proteins of interest (Figure 1). Once the recombinant genes encoding for the recombinant proteins are constructed, they are introduced into cells where the fusion proteins are expressed. When the two proteins of interest interact with each other, the cognate fragments are brought into proximity and refold to generate the reporter protein. PCAs that have been developed to the present are based on reporter proteins that generate signals in the form of survival, fluorescence or color. The first few PCAs established were designed for survival selection. These include murine Dihydrofolate Reductase Protein fragment Complementation Assay (mDHFR PCA) (Pelletier, Campbell-Valois et al. 1998), aminoglycoside phosphotransferase PCA, hygromycin B phosphotransferase PCA, and glycinamide ribonucleotide transformylase PCA (Michnick, Remy et al. 2000). Fluorescent and colorimetric PCAs have also been developed using mDHFR (Remy and Michnick 1999) and beta-lactamase (Galarneau, Primeau et al. 2002) as reporter proteins.

Some of the advantages of using PCA (Michnick 2001) are the following: First, PCA allows genes to be expressed in the relevant cellular context and recombinant proteins can appropriately undergo post-translational modifications. Second, PCA allows the direct detection of molecular interactions rather than via secondary events, such as transcriptional activation (Fields and Song 1989). Third, PCA allows interactions to occur in biologically

relevant compartments of cells (Remy and Michnick 2001). Finally, one of the most interesting features of PCA is that both fragments of the reporter protein do not spontaneously interact and fold. Fragment interaction/folding is induced only by the interaction of the interacting protein partners resulting in a clear "all or none, binary result" (Pelletier, Campbell-Valois et al. 1998).

In addition to the above-mentioned properties of PCA, it can also be regulated from the level of gene transcription (Zhang, Ma et al. 2004). Therefore, PCA can be regulated and controlled at two levels: first, at the level of gene expression and second, by protein-protein interactions. When one protein fused to a reporter fragment is expressed, no PCA signal can be detected. When both proteins, fused to respective reporter fragments, are expressed but no interaction occurs between them, no PCA signal can be detected (Pelletier, Campbell-Valois et al. 1998). Only when both proteins, fused to respective reporter fragments, are expressed and both proteins interact with each other, will a PCA signal be observed. The benefits of having two levels of regulatory points of PCA is the reason why we chose to develop the Binary Positive and Negative Selection strategy using PCA. Binary Positive and Negative PCA could be used for applications in positive selection assays (similar to the survival selection assay of mDHFR PCA) or negative selection assays (Figure 2). Negative selection PCA could also be referred to as death PCA since it consists of killing cells carrying interacting protein partners fused to fragments of the reporter protein. Applications of the death PCA will be further covered in this project since no previous death PCA has been developed.

1.3 Applications of the Binary Positive and Negative PCA using the Death PCA

Taking advantage of the multiples regulatory points of the Binary Positive and Negative PCA, various selection assays can be established. Here, we focus on some of the possible applications of the death PCA:

First, death PCA could be used to increase specificity of killing cancer cells. Many studies suggest that a toxic gene can be expressed under the control of a promoter of an over expressed gene in cancer cells in order to specifically kill the tumor cells (Nyati,

Sreekumar et al. 2002; Song, Kim et al. 2003). However, a promoter that is highly expressed in cancer cells could also be expressed in other tissues of an organism. For example, although the carcinoembryonic antigen (CEA) promoter is highly active in colon cancer cells, it is also highly expressed in the gastrointestinal cells of the colon (Eades-Perner, van der Putten et al. 1994; Chan and Stanners 2004). Human telomerase reverse transcriptase (hTERT) promoter is another tissue specific promoter that is highly expressed in tumor cells (Song, Kim et al. 2003). In normal cells, hTERT is expressed at very low level with the exception that it can also be expressed in leukocytes (Counter, Gupta et al. 1995) and stem cells. We hypothesize that using death PCA and two promoters of highly expressed genes in cancer cells will further increase the specificity for targeting tumor cells. This strategy consists of increasing the specificity of the toxic effect of suicide gene therapy to act in tumor cells that highly express both tumor marker genes and not in normal cells that express only one of the tumor marker genes.

Second, a death PCA could be used for screening for bioactive molecules. Survival selection strategies such as the yeast two-hybrids assay have successfully been demonstrated as an *in vivo* inhibitor screening method (Licitra and Liu 1996; Kato-Stankiewicz, Hakimi et al. 2002). However many survival selection strategies require a replica-plating step in order to recover clones. Replica-plating is time consuming and often leads to false-positives. A death selection tool would eliminate this step. Thus hundreds of thousands of molecules could be screened and only molecules with bioactive properties will stand out since they will disrupt the death selection mechanism and cells could grow and form colonies.

Finally, death PCA can be used to select for non-interacting mutants of an interacting-protein pair. For example, protein A and protein B interact with each other. However, we would like to identify mutant forms of protein A that do not interact with protein B. In this case, we could use mutagenesis and death PCA to screen for mutant forms of protein A that do not bind to protein B. We could generate a library of protein A mutants and use death PCA to screen for specific mutants. Since only non-interacting proteins fused to death PCA fragments allow cell growth, only cells expressing mutant forms of protein A that do not bind to protein B will be able to grow and form colonies. Plasmids carrying mutant

forms of protein A can be isolated from the surviving colonies and sequencing of the mutant forms of protein A can be done in order to identify mutations that change the properties of protein A.

1.4 Candidates for Death and Survival-Death PCA

Many proteins qualify to be a candidate for cell death selection assay or both survival and death selection assay. However, small and monomeric proteins with well-known structures are ideal candidates since they facilitate the process of developing a PCA. Unfortunately, not many "death" reporter proteins satisfy these conditions. Therefore we included small dimeric proteins in our list of candidate proteins for the development of the death PCA (Table I). There is always the possibility to engineer a small dimeric protein to become a functional monomeric protein such as seen in the case of GFP (Campbell, Tour et al. 2002).

In general, death reporter proteins can be categorized into two classes according to how they activate the cell death pathways. The first class consists of cytotoxic proteins that inhibit cell division by directly interacting with the cellular machinery. For example, bacterial toxin CcdB inhibits DNA gyrase (Bernard and Couturier 1992) and promotes cell death by causing DNA lesions such as double-stranded breaks in DNA (Loris, Dao-Thi et al. 1999). Diphtheria toxin A is another toxic protein that causes cell death by covalently attaching ADP-ribose to the N-1 of the imidazole ring of diphthamide in eukaryotic elongation factor 2 (EF-2) (Oppenheimer and Bodley 1981). The second class consists of proteins that indirectly inhibit cell division by activating a non-toxic compound to a toxic compound. The latter are also known as cytotoxic pro-drug converting enzymes. Herpes Simplex Virus-1 thymidine kinase (HSV1-TK), orotidine-5'-monophosphate decarboxylase (OMPdecase) and cytosine deaminase (CD) are some examples. Only proteins from this subset qualify for both survival and death assay and are therefore candidates for the development of the Binary Positive and Negative PCA. Among the candidates listed in this category, yeast cytosine deaminase (yCD) is an interesting candidate since the prodrug 5-fluorocytosine is widely available, inexpensive, and a small amount is required to cause

Table I: List of Death and Survival-Death PCA Candidates and Characteristics.

Protein Candidate	Molecular Weight	Type of PCA	Mechanism of Inhibition	Substrate	Effective Conc. for Growth Inhibition	Cell Type	
CcdB	11KDa	Death	Inhibit GyraseA	N/A	N/A	Bacteria	Negative Selection
DTA	55KDa	Death	Inhibit eukaryotic elongation factor 2	N/A	N/A	Mammalian cell	Negative Selection
yCD	17KDa	Survival-Death	Produce toxic metabolite 5-FU	5-FC	< 100 μg/ml for yeast	YCD KO yeast BCD KO bacterial Mammalian cell	Positive or Negative Selection
bCD	35KDa	Survival-Death	Produce toxic metabolite 5-FU	5-FC	N/A	YCD KO yeast BCD KO bacterial Mammalian cell	Positive or Negative Selection
HSV-TK1	42KDa	Survival-Death	Produce toxic metabolite PO4-Gancyclovir	Ganciclovir	> 1000 μg/ml for yeast	Bacteria Yeast Mammalian cell	Positive or Negative Selection
URA3	29KDa	Survival-Death	Produce toxic metabolite	5-FOA	> 1000 μg/ml for yeast	Yeast	Positive or Negative Selection

CcdB: Gene encoding for a toxin that inhibits bacterial DNA gyrase.
DTA: Diphtheria toxin A.
yCD: Yeast cytosine deaminase.
bCD: Bacterium cytosine deaminase.
HSV1-TK: Herpes Simplex Virus-1 thymidine kinase.
URA3: Gene encoding for orotidine-5'-monophosphate decarboxylase.

cytotoxicity in yeast and mammalian cells. HSV1-TK is also an interesting candidate for applications in mammalian cells since only 0.1 μM of the nucleoside analog, ganciclovir, is required to induce cell death (Black, Kokoris et al. 2001). However, more than 1000 μg/ml is required to temporarily inhibit cell growth in yeast (Wera, Degreve et al. 1999). Since our goal is to establish an assay that could be used in both yeast and mammalian cell systems, we chose to develop this survival and death PCA using yCD.

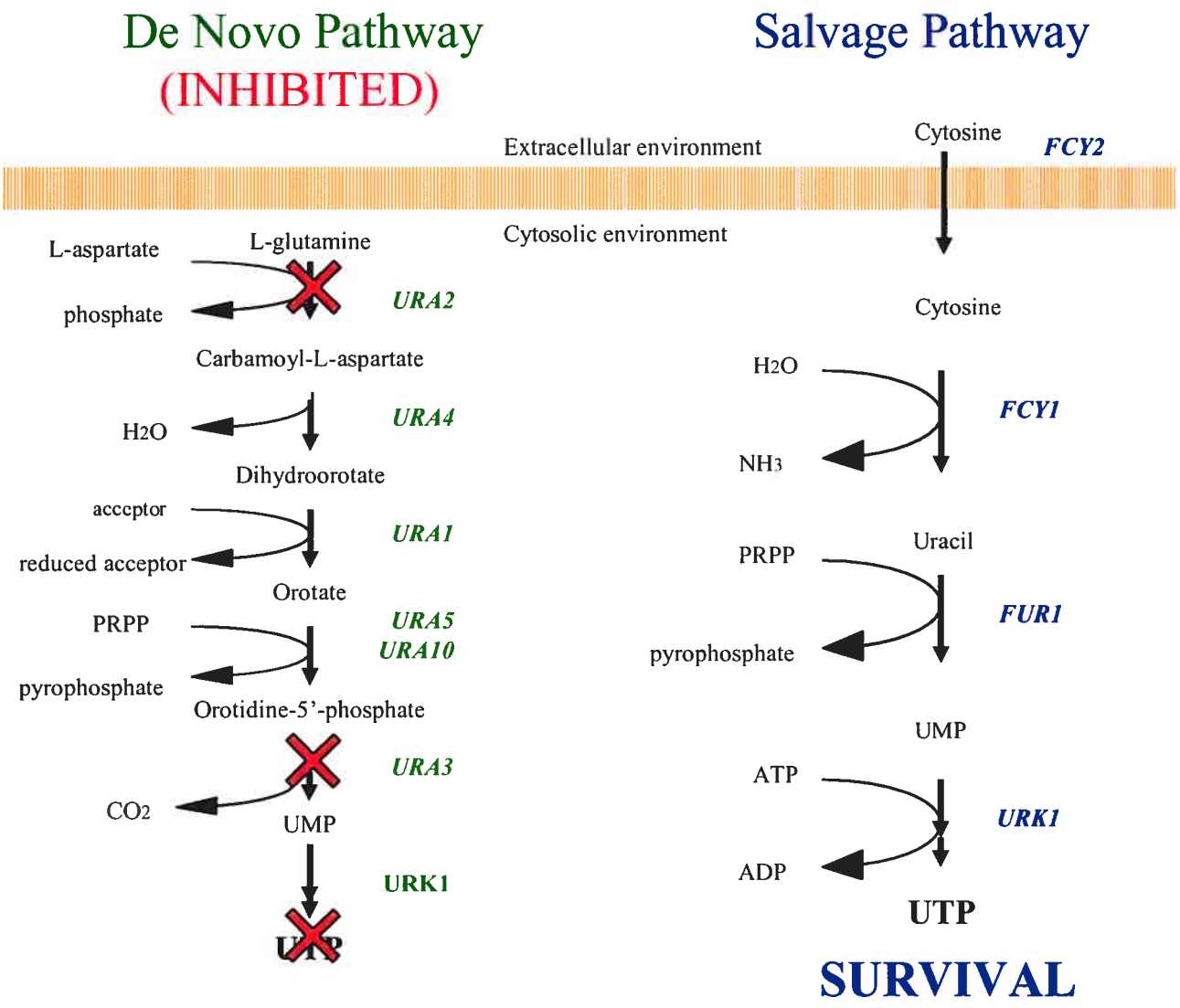
1.5 Yeast Cytosine Deaminase

Cytosine deaminase (CD) is an enzyme involved in the pyrimidine salvage pathway (Kurtz, Exinger et al. 1999) and was initially discovered in yeast and Escherichia coli (E. coli) in 1925 by Hahn and Schafer (Hahn and Schafer 1925). CD is not present in higher eukaryotic cells such as plant and mammalian cells (Nishiyama, Kawamura et al. 1985). The yeast cytosine deaminase (yCD), has been used as a reporter protein for both positive and negative selection as discussed below (Wei and Huber 1996; Gallego, Sirand-Pugnet et al. 1999; Xiaohui Wang, Viret et al. 2001).

a) yCD in pyrimidine salvage pathway and survival selection assay

In S. *cerevisiae*, yCD is encoded by the *FCY1* gene. The presence of this enzyme allows yeast to use environmental cytosine as a source of pyrimidine for the nucleotide pool (Figure 3) in the same way as bacteria (Grenson 1969). Cytosine enters into the cell using a purine-cytosine transporter encoded by the *FCY2* gene. In the cytosol, cytosine is deaminated to uracil by yCD (Erbs, Exinger et al. 1997). Once cytosine is converted to uracil, uracil can be phosphoribosylated to uridine 5'-monophosphate (UMP) by uracil phosphoribosyltransferase (encoded by the *FUR1* gene) (Kern, de Montigny et al. 1990). UMP can be further phosphorylated to become uridine 5'-triphosphate (UTP) by uridine kinase (encoded by the *URK1* gene). UTP can then be converted by thymidylate synthase (encoded by the *CDC21* gene) to thymidine monophosphate (TMP). Thus, yeast possesses a pyrimidine salvage pathway that allows it to recycle exogenous cytosine for the ribose and deoxyribose nucleotide pool.

Figure 3. Pyrimidine Salvage Pathway and Survival Selection Strategy in S. *cerevisiae*. When one of the key enzymes (encoded by *URA2* or *URA3* gene) of the pyrimidine *de novo* pathway is disrupted, the cell cannot produce uridine triphosphate (UTP). In order for the cell to survive, it must use the pyrimidine salvage pathway to import extracellular cytosine, deaminate cytosine to uracil by yCD (encoded by *FCY1* gene), and modify uracil to UTP. Proteins involved in this process are shown by their gene name in italics: aspartate transcarbamylase (*URA2*), dihydrooratase (*URA4*), dihydrooratate dehydrogenase (*URA1*), orotate phosphoribosyltransferase (*URA5 and URA10*), orotidine-5'-phosphate decarboxylase (*URA3*), uridine kinase (*URK1*), purine-cytosine transporter (*FCY2*), yeast cytosine deaminase (*FCY1*), uracil phosphoribosyltransferase (*FUR1*).

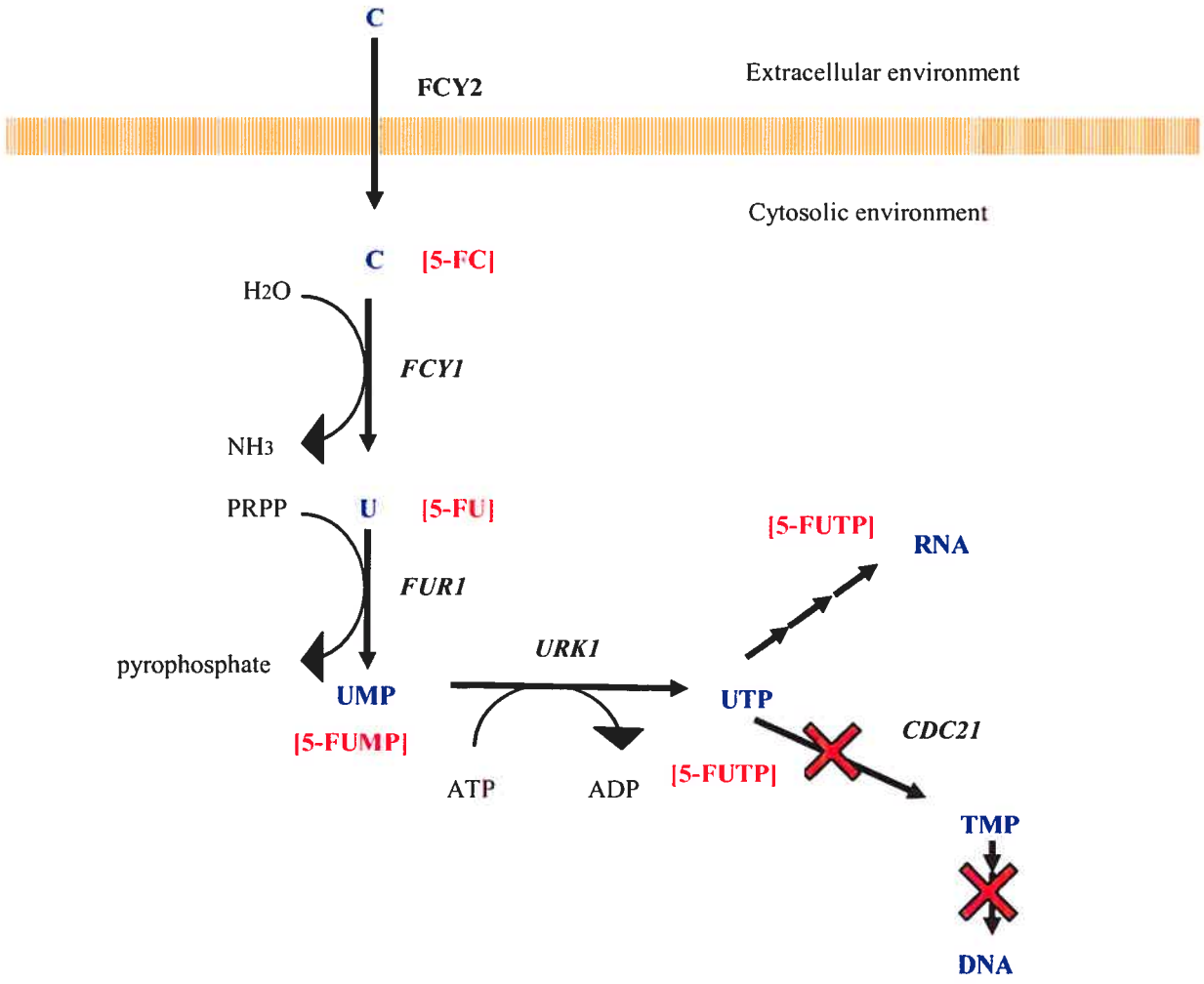

De Novo Pathway
(INHIBITED)
Salvage Pathway
Extracellular environment
Cytosolic environment
Cytosine
FCY2
L-aspartate
L-glutamine
phosphate
URA2
Carbamoyl-L-aspartate
H2O
URA4
Dihydroorotate
acceptor
reduced acceptor
URA1
Orotate
PRPP
pyrophosphate
URA5
URA10
Orotidine-5'-phosphate
CO2
URA3
UMP
URK1
Cytosine
H2O
NH3
FCY1
PRPP
Uracil
pyrophosphate
FUR1
UMP
ATP
ADP
URK1
UTP
SURVIVAL

In addition to relying on the pyrimidine salvage pathway, S. *cerevisiae* can synthesize ribose and deoxyribose nucleotides using the *de novo* pyrimidine synthesis pathway. However, when the *de novo* pyrimidine synthesis pathway is inhibited, cells become uracil auxotrophs and thus require the essential nucleoside uracil for survival. Under these conditions, the pyrimidine salvage pathway becomes the main source for generating uracil. Therefore, yCD can become an important regulatory enzyme that controls cell growth. This allows for the possibility of establishing a survival selection assay. Such a positive selection assay has been established in yeast (Erbs, Exinger et al. 1997) and in mammalian cells (Wei and Huber 1996). In yeast, OMPdecase is one of the key enzymes in uracil synthesis and pyrimidine *de novo* synthesis (Figure 3). Disruption of the URA3 gene will force the cell to use yCD to convert environmental cytosine to uracil for cell survival. In mammalian cells, gene knockouts in the pyrimidine *de novo* synthesis pathway also force cells to utilize the pyrimidine salvage pathway for survival. However, pyrimidine *de novo* synthesis can also be blocked by an inhibitor known as N-(phosphonacetyl)-L-aspartate (PALA). PALA inhibits aspartate carbamyl transferase (CADases), an enzyme in the carbamoyl-phosphate synthetase complex (Swyryd, Seaver et al. 1974). Mammalian cells treated with PALA will undergo apoptosis (Wei and Huber 1996) unless an exogenous cytosine deaminase enzyme (yCD) is introduced into the cell and cytosine is added as a supplement to the culture medium.

b) yCD Death Selection Assay

In addition to catalyzing the deamination of cytosine, yCD can also deaminate 5-methylcytosine and 5-fluorocytosine (5-FC) (Wang, Sable et al. 1950). 5-FC was initially synthesized in 1957 in prospective as an antitumor agent but instead was found to have utility as an anti-fungal agent (Grunberg, Titsworth et al. 1963). yCD can deaminate 5-FC, a relatively non-toxic compound, to 5-Fluorouracil (5-FU), a toxic compound (Figure 3 in brackets). 5-FU can be ribosylated to 5-fluorouridine monophosphate (5-FUMP) by uracil phosphoribosyltransferase (*FUR1* gene). 5-FUMP is further phosphorylated by uridine kinase (*URK1* gene) to become 5-fluorouridine triphosphate (5-FUTP). 5-FUTP inhibits cell growth by incorporating into RNA (Fang, Hoskins et al. 2004; Lum, Armour et al. 2004) and directly inhibiting thymidylate synthase (*CDC21* gene), an enzyme that controls

Figure 4. Pyrimidine Salvage Pathway and 5-Fluorocytosine Death Assay in S. *cerevisiae*. Enzymes involved in this process are shown by their gene name in italics. Environmental cytosine is transported into the cell by the purine-cytosine transporter (*FCY2*). Yeast cytosine deaminase (*FCY1*) deaminates cytosine (C) to generate uracil (U). Uracil phosphoribosyltransferase (*FUR1*) phosphoribosylates uracil to uridine monophosphate (UMP). UMP is phosphorylated by uridine kinase (*URK1*) to uridine diphosphate and uridine triphosphate (UTP). UTP can incorporate into RNA and serve as a substrate for thymidylate synthase (*CDC21*) for the *de novo* synthesis of thymidine monophosphate (TMP). 5-Fluorocytosine (5-FC) can also be processed by enzymes of the pyrimidine salvage pathway and allow the establishment of a death assay. 5-FC and its derivatives are shown in brackets. 5-FUTP is the toxic compound that causes inhibition of cell growth. 5-FUTP can incorporate into RNA and inhibit thymidylate synthase.


C
FCY2
Extracellular environment
Cytosolic environment
C [5-FC]
H2O
FCY1
NH3
PRPP
U [5-FU]
FUR1
pyrophosphate
UMP
[5-FUMP]
URK1
ATP
ADP
UTP
[5-FUTP]
RNA
CDC21
[5-FUTP]
TMP
DNA

DNA synthesis (Parker and Cheng 1990; Longley, Harkin et al. 2003). In S. *cerevisiae*, the effects of yCD and 5-FC resulted in morphological changes preventing daughter cells to separate from the mother cells (Zhang, Zhang et al. 2002). Hence, yCD and 5-FC can be used as a selection tool for targeting cell death. This negative selection has been suggested for a plasmid shuffling assay by Erb *et al.* in 1997 and is currently being explored for applications in gene therapy.

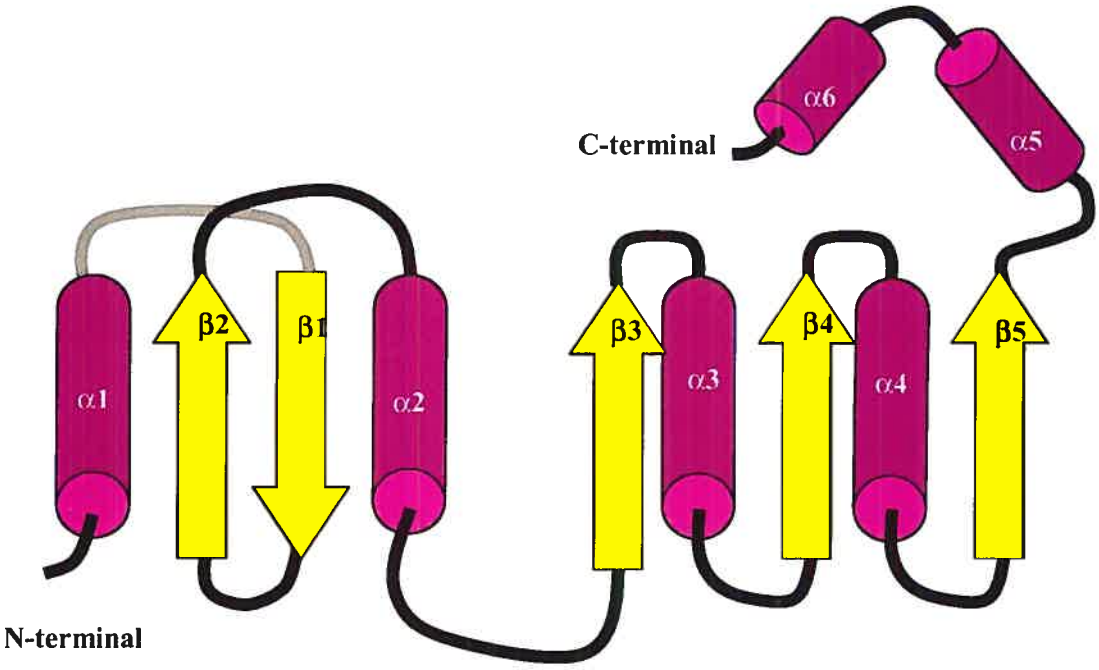
1.6 Selection of a cellular system for the development of the Binary Positive and Negative PCA

The selection of the cellular system for establishing a Binary Positive and Negative PCA is dependent on the selection of the reporter protein. The objective is to select a system that is amenable to monitoring the signal of the PCA. Wild-type strain of S. *cerevisiae* has a gene that encodes for cytosine deaminase (yCD). However, many yCD knockout strains of S. *cerevisiae* have been reported in small scale (Erbs, Exinger et al. 1997) and large scale (Giaever, Chu et al. 2002) studies. The latter large scale gene disruption project utilized S. *cerevisiae* strain BY4741 (*MATa ura3Δ0 leu2Δ0 his3Δ1 met5Δ0*) and BY4742 (*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*) for their gene disruption project. Since the URA3 gene is disrupted in both BY4741 and BY4742 strains, the pyrimidine *de novo* synthesis pathway can be blocked by removing cytosine from the culture medium. yCD knockout cells cannot grow under these conditions. yCD becomes the regulatory enzyme for the pyrimidine salvage pathway and can regulate cell survival. These yCD knockout strains of BY4741 and BY4742 can be used for the development of our yCD PCA since both the survival and death selection assays can be tested in these strains.

1.7 Approaches for establishing the Binary Positive and Negative yCD PCA

The approach for developing yCD PCA consisted of carefully analyzing the structure of the protein and selecting specific sites for fragmentation. Furthermore, in order to increase the possibility of finding the best yCD fragments that can generate highest yCD PCA activity, a fragment shuffling experiment can be done.

Figure 5. yCD topology. yCD monomer is composed of six alpha (α) helices and five beta (β) strands. The first α-helix (α1) is found at the N-terminal of the protein followed by a β-hairpin, (β2, β3), a second α-helix (α2), a Rossmon fold (β3-α3-β4-α4-β5), and two short helices (α5-α6). The five β-strands form a β-sheet sandwiched between α-helices (α1 and α5) on one side and α-helices (α2, α3, α4, and α6) on the other side.


C-terminal
α6
α5
β2
β1
β3
β4
β5
α1
α2
α3
α4
N-terminal

a) Molecular Characteristics of yCD

The structure of yCD has recently been solved (Ireton, Black et al. 2003; Ko, Lin et al. 2003). yCD differs significantly from bacterial cytosine deaminase (bCD) in terms of quaternary structure, primary amino acid sequence, molecular mass, and relative substrate specificities and affinities. yCD is a homodimer where the 17.5 KDa monomers are arranged in a 2-fold symmetry axis. The dimer is formed from the association of the two monomers in a head-to-tail orientation. Each monomer forms a compact domain composed of six alpha (α) helices and five beta (β) strands (Figure 5). The five β-strands form a β-sheet sandwiched between α-helices (α1 and α5) on one side and α-helices (α2, α3, α4, and α6) on the other side. There is one active site present per subunit. The active site involves amino acid histidine-62, glutamate-64, cysteine-91, and cysteine-94. Each active site can bind a tetrahedral catalytic zinc ion, which helps to coordinate the substrate and participate in an acid/base catalysis mechanism. Amino acids histidine-62, cysteine-91 and cysteine-94 are also involved in the coordination of the zinc molecule.

b) Fragmentation of a Reporter Protein

The fragmentation sites were chosen in regions of the enzyme not involved in secondary structure elements such as alpha-helices or beta-strands of the protein. Cutting reporter proteins in loop regions connecting one secondary structure element to another is favored. In the case that the reporter protein is an enzyme, we select for loop regions not involved in the active site of the enzyme. Cutting within secondary structure elements could interfere with the refolding of the reporter protein. Thus, the idea is to fragment the reporter protein in such a way that it could easily refold to its functional structure resembling the native protein.

c) Optimization of yCD PCA Activity by Fragments Shuffling

It has previously been shown that two fragments of an enzyme containing overlapping amino acid residues could rearrange to generate a functional enzyme (Taniuchi and Anfinsen 1971; Ostermeier, Nixon et al. 1999). In addition, it has also been known that

elimination of some residues in a peptide fragment could increase or decrease the activity of an engineered protein (Ostermeier, Nixon et al. 1999). By shuffling yCD fragment 1 versus yCD fragment 2 of the different cut sites, different PCA combinations can be screened in order to increase the activity of yCD PCA.

1.8 Specific aims

The goal of this project is to develop a Binary Positive and Negative PCA using yeast cytosine deaminase (yCD PCA). In brief, this study consisted of: 1) fragmenting yCD to generate the PCA; 2) testing yCD fragments for yCD PCA activity using the death and survival assay as well as improving yCD PCA activity; and 3) demonstrating a possible application of the Death PCA in a mammalian cell system.

CHAPTER 2: MATERIALS AND METHODS

2.1 Yeast genomic DNA, yCD knockout yeast strains, and substrates

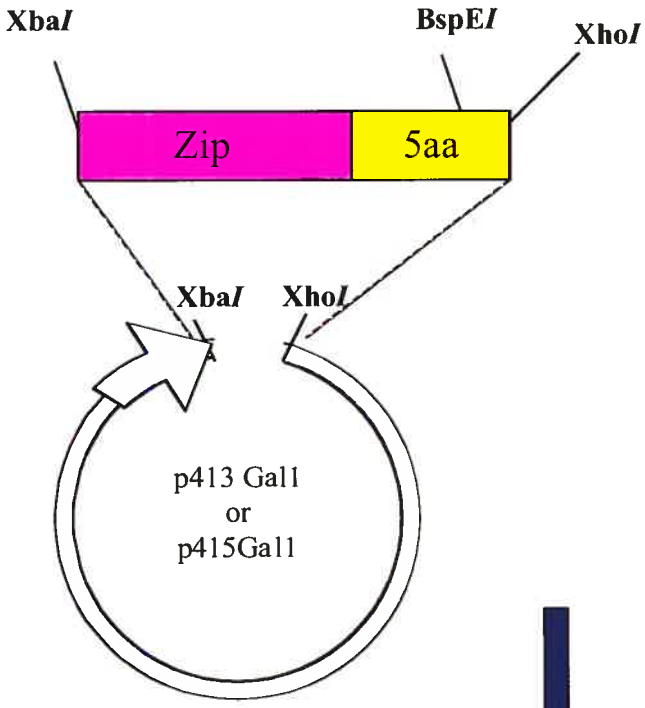
S. *cerevisiae* strain BY4743 (diploid *ura3Δ0 leu2Δ0 his3Δ1 met5Δ0 lys2Δ0*) was used for isolation of genomic DNA containing two wild-type copies of the yCD gene. S. *cerevisiae* BY4741 yCD knockout and BY4742 yCD knockout, generated as part of the yeast gene knockout collection (Giaever, Chu et al. 2002), were used for yCD PCA since their genomic copy of yCD have been disrupted. All yeast strains were gifts from Dr. Howard Bussey. YPD (yeast extract, peptone, and dextrose) and synthetic defined medium with amino acids complementation (SDC) were prepared as described by Guthrie et al. (Guthrie C, Fink GR 1991). YPR062w knockout strains were propagated in medium containing 200 μg/ml of Geneticin® (G418) purchased from Invitrogen (Burlington, Ontario). 5-FC and cytosine [purchased from Sigma (Oakville, Ontario)] were diluted in water to a concentration of 10 mg/ml and kept as stock solution.

2.2 yCD PCA development in yeast

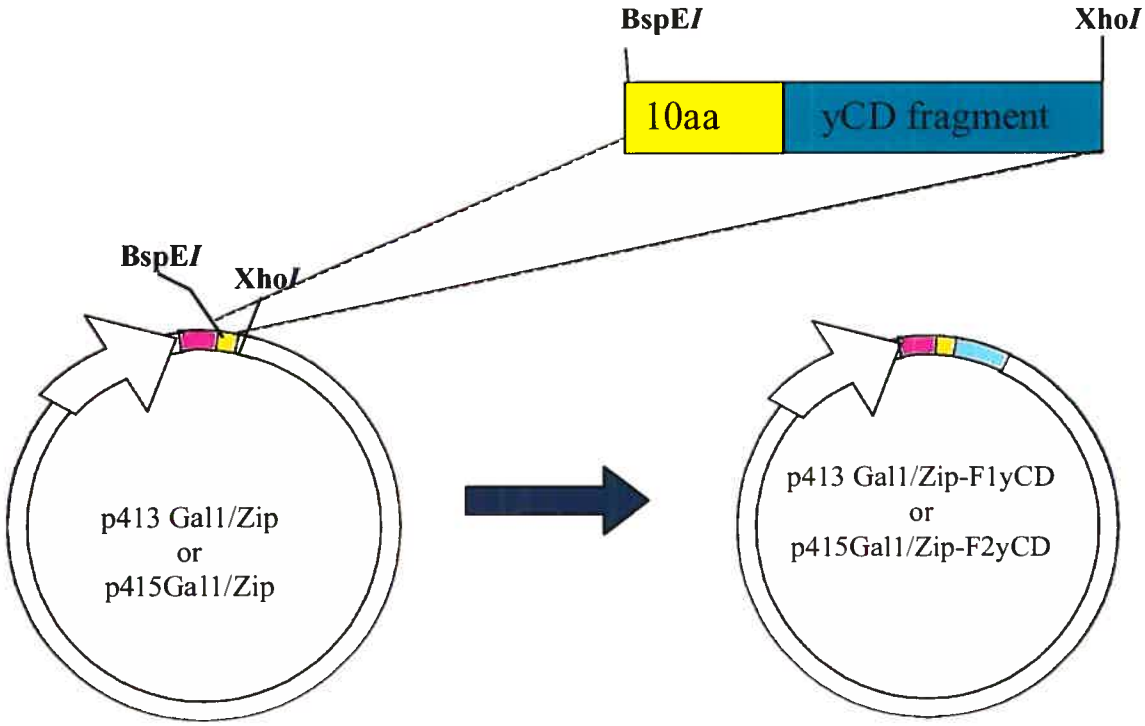
Yeast expression vectors p413Gal1 and p415Gal1 (Mumberg, Muller et al. 1995), used for yCD PCA development, were gifts from Dr. Howard Bussey. First, GCN4 leucine zipper and one third of the linker sequence coding for amino acids GGGGS was amplified by polymerase chain reaction (PCR) with pfu polymerase, purchased from Fermentas (Burlington, ON), from the plasmid pcDNA3.1-Zip-[F1.2] mDHFR (Remy and Michnick 1999) using the following primers: 5'-cgc tctaga ggg ATGAACACTGAAGCCGCCAGGCG-3' and 5'-ccg ctcgag cta tccgga gccaccgccacc GCGTTCGCCAACTAATTTC-3'. GCN4 leucine zipper was cloned into the multiple cloning sites of p413Gal1 and p415Gal1 vectors at the XbaI and XhoI restriction sites. A unique BspEI site was added as part of the linker sequence for cloning DNA fragements downstream of the GCN4 leucine zipper sequence (Figure 6). The vectors carrying these GCN4 leucine zipper sequence are named p413Gal1-Zip and p415Gal1-Zip.

Figure 6. yCD PCA construction scheme. First, the gene of interest is cloned into the vector, for example GCN4 coil-coil leucine zipper (Zip) sequence cloned into the multiple cloning sites with a flexible linker and **BspE*I*** of p413Gal1 or p415Gal1vectors using the **Xba*I*** and **Xho*I*** sites. Next, yCD or yCD fragments are cloned downstream of the Zip sequence using **BspE*I*** and **Xho*I*** sites.

Step1: Insert Zip and 5 amino acids (5aa) sequence with **BspE*I*** restriction site in multiple cloning sites of vector using **Xba*I*** and **Xho*I*** sites.



Step2: Insert 10 amino acids sequence and yCD fragment using **BspE*I*** and **Xho*I*** sites.



yCD gene and yCD fragments were amplified from S. *cerevisiae* strain BY4743 genomic DNA using pfu polymerase (Fermentas, Burlington, Ontario) with the remaining linker sequence coding for amino acids GGGGSGGGGS. Conditions used for PCR were the following: 95°C (5 min), 25 cycles of [95°C (1 min), 55°C (1 min), 72°C (1 min)], 72°C (3 min). The primers used for amplifying yCD and yCD fragments are listed in Table II. These PCR products were cloned into p413Gal1 and p415Gal1vectors downstream of GCN4 leucine zipper sequence using BspEI and XhoI restriction sites and T4 DNA ligase purchased from Fermentas (Burlington, Ontario).

2.3 Yeast Cell Transformation

Competent yeast cells were prepared and stored at –80°C (Knop, Siegers et al. 1999). In brief, *MAT***a**, *MAT*α, and diploid yCD knockout yeast colonies were inoculated for 6 hours in 5 ml of YPD medium with 200 μg/ml G418. These pre-cultures were used to start a 100 ml culture for overnight incubation. The next day, when the culture reached OD_{600} 0.5 to 0.7, cells were harvest by centrifugation at 1500 rpm for 5 min at room temperature (RT). The pellets were washed with 5 ml of sterile water, centrifuged for 5 min at 1500 rpm, and washed with 5 ml of Sorbitol Buffer (1 M sorbitol, 1 mM EDTA, 10 mM Tris pH 8.0, 100 mM LiOAc). Pellets were resuspended in 720 μl of Sorbitol Buffer and 80 μl of Salmon Sperm DNA at 10 mg/ml stock solution purchased from Sigma (Oakville, Ontario).

Yeast cell transformation was performed by mixing 100 ng of plasmid DNA with 25 μl of frozen competent yeast cells in a 1.5 ml microtube. 150 μl of PLATE solution (40% PEG 4000, 100 mM Lithium Acetate, 10 mM Tris pH 7.5 and 0.4 mM EDTA) was added. The sample was mixed and allowed to incubate for 30 min at RT. 20 μl of Dimethylsulfoxide (DMSO), purchased from Fisher Scientific (Fairlawn, NJ), was added, and incubated at 42 °C for a 20 min heat shock. Cells were pelleted by centrifugation at 2000 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in 200 μl of PLATE solution. 20 μl of the sample was plated on synthetic defined medium with amino acid complementation for selection (6-well plate) and allowed to incubate at 30 °C for 3 days.

Table II. Primers for generating yCD fragments at 7 cut sites and yCD full-length enzyme. Nucleotide sequences in uppercase correspond to sequences that directly anneal on yCD gene, nucleotide sequences in lowercase from the sense primers column correspond to flexible linker sequences, and underligned nucleotide sequences correspond to restriction sites.

Gene Name	Sense Primers	Anti-sense Primers
F1yCDcut1	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct ATGGTGACAGGGGGAATGGC-3'	5'-ccg ccc ccg ctcgag cta CTTTTGAAATCTCATGTT-3'
F1yCDcut2	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct ATGGTGACAGGGGGAATGGC-3'	5'-ccg ccc ccg ctcgag cta TCTCCCACAGTTTTCCAAAGTGGAG-3'
F1yCDcut3	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct ATGGTGACAGGGGGAATGGC-3'	5'-ccg ccc ccg ctcgag cta GCCCTCTAATCTCCCACAG-3'
F1yCDcut4	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct ATGGTGACAGGGGGAATGGC-3'	5'-ccg ccc ccg ctcgag cta TTTGCCCTCTAATCTCCC-3'
F1yCDcut5	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct ATGGTGACAGGGGGAATGGC-3'	5'-ccg ccc ccg ctcgag cta TTTGTACACTTTGCCCTC-3'
F1yCDcut6	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct ATGGTGACAGGGGGAATGGC-3'	5'-ccg ccc ccg ctcgag cta GTTCTCACCGACAACACA-3'
F1yCDcut7	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct ATGGTGACAGGGGGAATGGC-3'	5'-ccg ccc ccg ctcgag cta AACAACAACAACCTCGTG-3'
F2yCDcut1	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GGATCCGCCACACTACAT-3'	5'-ccg ccc ccg ctcgag cta CTCACCAATATCTTCAAACC-3'
F2yCDcut2	5'-ggc tccgga ggt gga ggt tct gga ggt TTAGAGGGCAAAGTGTACAAAG-3'	5'-ccg ccc ccg ctcgag cta CTCACCAATATCTTCAAACC-3'
F2yCDcut3	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct AAAGTGTACAAAGATACCAC-3'	5'-ccg ccc ccg ctcgag cta CTCACCAATATCTTCAAACC-3'
F2yCDcut4	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GTGTACAAAGATACCACT-3'	5'-ccg ccc ccg ctcgag cta CTCACCAATATCTTCAAACC-3'
F2yCDcut5	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GATACCACTTTGTATACG-3'	5'-ccg ccc ccg ctcgag cta CTCACCAATATCTTCAAACC-3'
F2yCDcut6	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GTTAATTTCAAAAGTAAGGGC-3'	5'-ccg ccc ccg ctcgag cta CTCACCAATATCTTCAAACC-3'
F2yCDcut7	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct GACGATGAGAGGTGTAAA-3'	5'-ccg ccc ccg ctcgag cta CTCACCAATATCTTCAAACC-3'
C-ter yCD	5'-ggc tcc gga ggt gga ggt tct gga ggt ggc gga tct ATGGTGACAGGGGGAATGGC-3'	5'-ccg ccc ccg ctcgag cta TCTCCCACAGTTTTCCAAAGTGGAG-3'

2.4 Protein Expression in Yeast

Colonies were inoculated in 1 ml of synthetic defined medium (6.7 g/L of yeast nitrogen base without ammonium sulphate and amino acids [purchased from Bioshop]), 1 g/L monosodium glutamic acid, 2 g/L amino acid drop-out lacking methionine, lysine, histidine, or leucine, 200 μg/ml G418, and 2% raffinose (SC -met -lys -his or –leu +G418). The cultures were grown overnight and then induced with 2% galactose for 24 hrs. Cells were collected and prepared for protein extraction using Lyticase method (Sambrook J. 1989). The cell pellet was washed with Phosphate-Buffered Saline (PBS). The pellet was resuspended in Stabilizing Buffer A [1 M sorbitol, 10 mM MgCl2, 2 mM dithiothreitol (DTT), 50 mM potassium phosphate pH 7.8, 100 μg/ml, and phenylmethylsulfonyl fluoride (PMSF)] and incubated at 30 °C for 10 min. The sample was pelleted, resuspended in Stabilizing Buffer B (1 M sorbitol, 10 mM MgCl2, 2 mM DTT, 25 mM potassium phosphate pH 7.8, 25 mM sodium succinate pH 5.5, 100 μg/ml PMSF), and incubated at 30 °C for 2 min. Then 10 μl of Lyticase was added to the sample and incubate at 30 °C for 30 min. Protoplasts were collected by centrifugation at 12 000 rpm for 10 min at 4 °C, resuspended in 100 μl No-salt lysis buffer (50 mM HEPES pH 7.0, 1% NP-40, 1 μg/ml aprotinin, and 100 μg/ml PMSF), and incubated for 30 min on ice. Protein concentration was determined using Bio-Rad DC Protein Assay [purchased from Bio-Rad (Hercules, CA)] since 1 % NP-40 was used in the lysis buffer.

For western blot analysis, 30 μg of protein extracts were loaded per well onto a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins from the acrylamide gels were transferred onto PVDF membranes. The membranes were blocked with 5% milk in Tris-NaCl Buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.2% Tween) overnight. Membranes were probed at RT for one hour with anti-yCD purchased from Biogenesis (Poole, England) at a 1/1000 dilution and with anti-sheep HRP purchased from Upstate cell signaling solutions (Lake Placid, NY) at a 1/5000 dilution. Membranes were washed 3 times 10 min with Tris-NaCl Buffer after each antibody incubation. Membranes were revealed with Western Lightning® Chemiluminescence Reagent Plus purchased from PerkinElmer (Boston, MA). Membranes were stripped with stripping solution purchased from Promega (Madison, WI) and re-

blocked with 5% milk in Tris-NaCl Buffer for 30 min at RT. Membranes were probed at RT for one hour with anti-yeast 3-phosphoglycerate kinase (anti-PGK) purchased from Molecular Probes (Eugene, Oregon) at a 1/1000 dilution, washed 3 times for 10 min with Tris-NaCl Buffer, probed with anti-mouse HRPO purchased from BD Biosciences (San Diego, CA) at a 1/5000 dilution, and revealed with Western Lightning® Chemiluminescence Reagent Plus.

2.5 5-FC Death Assay on Liquid and Solid Medium

Yeast colonies were inoculated overnight in 1 ml of SDC-met-lys-his-leu + G418 + 2% raffinose. The next day, 20 µl of the culture was transferred to 1 ml of SDC-met-lys-his-leu + G418 + 2 % raffinose and 2 % galactose for a 6 hrs induction at 30 °C.

For 5-FC death assay in liquid medium, either 500 or 5000 cells were transferred to a 96-well plate with selection medium containing 0, 100 or 1000 µg/ml of 5-FC. Amount of cells were determined based on optical density measurement at 600 nm ($OD_{600\ nm}$). $OD_{600\ nm}$ of 1 equals to 10^6 cells per ml. Samples were incubated at 30 °C with shaking for 1 to 2 days. After the incubation time, 200 µl samples were transferred to a 96-well plate for measuring $OD_{600\ nm}$ with Spetramax 190 from Molecular Devices (Sunnyvale, CA). The percentage of relative growth inhibition was calculated as follows: [($OD_{600\ nm}$ non-treated cells – $OD_{600\ nm}$ treated cells) / ($OD_{600\ nm}$ non-treated cells)] * 100.

For 5-FC death assay on solid medium, around 5000 cells were transferred to 1 ml of SDC-met-lys-his-leu + G418 + 2% raffinose and 2% galactose (with and without 5-FC) for an 18 hrs pre-incubation at 30 °C shaking. After the pre-incubation period, 10 µl of the samples were plated on solid selection medium: SDC-met-lys-his-leu + 2% Agar + G418 + 2% raffinose and 2% galactose (with and without 5-FC). Plates were incubated at 30 °C for either 2, 3, or 6 days.

2.6 Survival Assay on Solid Medium

Yeast colonies were inoculated overnight in 1 ml of SDC-met-lys-his-leu + G418 + 2% raffinose. The next day, 20 µl of the culture was transferred to 1 ml of SDC-met-lys-

his-leu + G418 + 2% raffinose and 2% galactose for a 6hrs induction at 30 °C. 10 μl of the samples were plated solid selection medium: SDC-met-lys-his-leu + 2% agar + G418 + 2% raffinose and 2% galactose (with and without cytosine). Plates were incubated at 30 °C for either 2, 3, or 6 days.

2.7 Mammalian Cell Lines and Reagents

Mammalian HEK293 was a gift from Dr. Guy Boileau's laboratory. Mammalian colon cancer cell lines CaCo-2 and LoVo were generously provided by Dr. Clifford Stanners' laboratory. Mammalian 6, 12, 96-well culture plates are treated for cell culture and are purchased from Corning COStar (Acton, MA). Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium alpha-Medium (α-MEM), Dulbecco's Phosphate-Buffered Saline (PBS) and Trypsin-EDTA are from Invitrogen (Burlington, Ontario). Fetal Bovine Serum (FBS) is provided by Wisent (Saint-Jean-Baptiste de Rouville, Quebec). Transfecting reagent Fugene 6 is from Roche Diagnosics (Basel, Switzerland). Zeocin™ was purchased from Invitrogen (Burlington, ON)

2.8 Construction of Mammalian Cell Promoter Characterization Vectors

Primers used to amplify tissue specific promoters are listed in Table III. Carcinoembryonic antigen promoter (CEAp from -407bp to –43bp), CEA enhancer (CEAe from -6.1kb to –4kb), CC6 promoter (CC6p from -281bp to –2bp), and CC6 enhancer (CC6e from-1.24kb to –0.585kb) were amplified with pfu polymerase (Fermentas) from LoVo cell line genomic DNA using the following PCR condition: 95°C (5min), 25 cycles of [95°C (1min), 62°C (1min), 72°C (1min)], 72°C (3min). Human telomerase reverse transcriptase promoter (hTERTp) was amplified with pfu polymerase (Fermentas) from LoVo cell line genomic DNA using the following PCR conditions: 95°C (5min), 25 cycles of [95°C (1min), 60°C (30sec), 72°C (30sec)], 72°C (3min). The PCR reaction was supplemented with 250 μg of bovine serum albumin and 0.1 M of betaine both purchased from Sigma (Oakville, ON). CEAp, CC6p and hTERTp were cloned into pGL3prom,

Table III. Primers for amplifying tissue specific promoters from genomic DNA of LoVo colon cancer cell line.

Promoter Name	Sense Primers	Anti-sense Primers
CEAp	5'-cccggg ctcgag CCCGGGACCCTGCTGGGTTTC-3'	5'-cccggg aagctt GAGTTCCAGGAACGTTTTGTC-3'
CEAe	5'- cccggg acgcgt GGTTACATTACAAAGTGGAAT-3'	5'-gggggg ctcgag CGGCTCACTGCAACCTCTGCCTC-3
CC6p	5'-cccggg ctcgag CTTGCTTCTCAGAGCATCTTC-3'	5'-cccggg aagctt GGTCTCTGCTGTCTTCTCTGT-3'
CC6e	5'-cccggg acgcgt CACAGCAATAAACACAATGAT-3'	5'-gggggg ctcgag TTATGTGACTCTAATTTCCTG-3'
hTERTp	5'-atatat ctcgag AGTGGATTCGCGGGCACAGA-3'	5'-atatat aagctt AGGGCTTCCCACGTGCGCAG-3'

purchased from Promega (Madison, WI) vector by removing the original SV40promoter with XhoI and HindIII. The new vectors are subsequently named pGL3/CEAp, pGL3/CC6p and pGL3/hTERTp. CEAe and CC6e were cloned upstream of CEAp and CC6p using MluI and XhoI sites generating plasmids: pGL3/CEAep and pGL3/CC6ep.

In order to generate the negative control vector, the original pGL3prom vector was digested with XhoI and HindIII to remove the SV40 promoter. The linearized vector was treated with Mung Bean Nuclease [purchased from NEB (Beverley, MA)] to remove the 5'nucleotide extensions and ligated with T4 DNA Ligase [purchased from Fermentas (Burlington, Ontario)]. This new promoterless vector was named pGL3noProm. pGL3/CEAp, pGL3/hTERTp, and pGL3noProm vectors were confirmed with sequencing.

2.9 Mammalian cell Promoter assay vector

2 $X10^5$ HEK 293 and CaCo2 cells were seeded in 12-well plates purchased from Corning (Acton, MA). The following day, cells were co-transfected respectively with 1 μg of pGL3/CEAp, pGL3/CEAep, pGL3/CC6p, pGL3/CC6ep, pGL3/hTERTp, pGL3prom, and pGL3noProm plasmid DNA and 100 ng of pRL-CMV [purchased from Promega (Madison, WI)] using Fugene (Roche) transfecting reagent. pRL-CMV is a plasmid that carries the R*enilla* luciferase gene under the Cytomegolovirus (CMV) promoter. 24 hours after the transfection, cells from each well of the 12-well plate were trypsinized and re-plated in 96-well clear-bottom white plate. 48 hours after the transfection, cells from the same transfection were assayed for firefly luciferase and R*enilla* luciferase activity. Firefly luciferase activity was assayed by using Promega Bright-Glo™ Luciferase Assay kit, purchased from Promega (Madison, WI), for quantification of firefly luciferase activity. In brief, 100 μl of the substrate was directly added to cells in the 96-well plate and cells were lysed. Luminescence signal was acquired for 30 sec using PACKARD FUSION MULTIDETECTION PLATE READER purchased from PerkinElmer (Woodbridge, Ontario). Activity of R*enilla* luciferase was quantified by the addition of 1 μM of Coelenterazine [purchased from Biotium, (Hayward, CA)] and luminescence signal was acquired for 30 sec. Results of the firefly luciferase activity were normalized to results of the R*enilla* luciferase activity. Each plasmid was tested in three to four different

transfection experiments. Data from the promoter assay varied relative to differences in transfection efficiency from each transfection. Therefore, results of only one transfection experiment are represented since the overall trend of the promoter activity assay is the same.

2.10 Mammalian Expression Vectors Construction

Mammalian expression vectors pcDNA3.1neo and pcDNA3.1zeo were purchased from Invitrogen. These expression vectors were chosen for their antibiotic resistance genes, which were used to generate stable cell lines. Zip-yCD, and Zip-[F1]yCDcut4 were amplified from yeast expression vectors (p413Gal1 series) and sub-cloned into pcDNA3.1neo using HindIII and XbaI. Zip-yCD and Zip-[F2]yCDcut4 were amplified from yeast expression vectors (p415Gal1) and sub-cloned into pcDNA3.1zeo using HindIII and XbaI. This generates the following vectors: pcDNA3.1neo/Zip-yCD, pcDNA3.1neo/Zip-[F1]yCD cut 4, pcDNA3.1zeo/Zip-yCD, and pcDNA3.1zeo/Zip-[F2]yCD cut4. The GCN4 leucine sequence was replaced by the library optimized WinZipA1 (WinA) or WinZipB1 (Win B) leucine zipper (Pelletier, Arndt et al. 1999). WinA has the following amino acid sequence: STTVAQLEEKVKTLRAQNYELKS RVQRLREQVAQLAS. WinB has the following amino acid sequence: STSVDELQAEVDQL QDENYALKTKVAQLRKKVEKLSE. WinA was generated by overlapping oligonucleotide fragments and PCR using oligos: f-WinA: 5'-GGGAAGCTTACCATGTCCACCACCGTGGCCCAGCTGGAGGAAAAGGTGAA AACCCTGAGAGCCCAGAACTACGAGCTGAAGTCC-3' and r-WinA: 5'-GCCCAG AACTACGAGCTGAAGTCCAGAGTGCAGAGGCTGAGAGAACAAGTCGCCC AGCTGGCCTCCGGTGGCGGTGGCTCCGGA TAG -3'. WinB was generated by overlapping oligonucleotide fragments and PCR using oligos: f-WinB: 5'-GGGAAGCTTACCATGTCCACCTCCGTGGACGAGCTGCAGGCCG AGGTGGACCAGCTGCAGGACGAGAACTACGCCCTGAAGACC-3' and r-WinB: 5'-GACGAGAACTACGCCCTGAAGACCAAGGTGGCTCAGCTGAGAAAGAAGGTGG AGAAGCTGTCCGAAGGTGGCGGTGGCTCCGGATAG-3'. PCR was performed at 95°C (5 min), [95°C (1 min), 55°C (1 min), 72°C (1 min)] for 25 cycles, 72°C (3 min) with Pfx AccuPrime from Invitrogen (Burlington, Ontario). Giving rise to the following set of

vectors: pcDNA3.1neo/WinA-yCD, pcDNA3.1neo/ WinA-[F1]yCD cut 4, pcDNA3.1zeo/ WinB -yCD, and pcDNA3.1zeo/WinB-[F2]yCD cut4. All of the above vectors were confirmed by sequencing and have the original CMV promoters of the pcDNA3.1neo and pcDNA3.1zeo.

In order to obtain tissue specific promoters, CEAp, and hTERTp were digested from pGL3/CEAp and pGL3/hTERTp with MluI and HindIII restriction sites and sub-cloned into all previously constructed pcDNA3.1neo or pcDNA3.1zeo based vectors by removing the CMV promoter with MluI and HindIII sites. This generated the following plasmids: pcDNA3.1neo/CEAp/WinA-yCD, pcDNA3.1neo/CEAp/WinA-[F1]yCD cut 4, pcDNA3.1zeo/hTERTp/WinB-yCD, and pcDNA3.1zeo/ hTERTp/WinB-[F2]yCD cut4.

[F2]yCD cut1 was sub-cloned downstream of promoter specific vectors using BspEI and XhoI to generate pcDNA3.1zeo/WinB-[F2]yCD cut1, and pcDNA3.1zeo/hTERTp /WinB-[F2]yCD cut1.

2.11 Generating Stable Transfectants

2.5 X 10^5 cells were seeded in 12-wells plates 24 hrs prior to transfection. Cells were transfected with 1 μg of plasmid DNA. The following combinations of plasmids were used: a) CEAp /WinA-[F1]yCD cut4 and hTERTp/WinB-[F2]yCD cut4, b) CMVp /WinA-[F1]yCD cut4 and CMVp/WinB-[F2]yCD cut4, c) hTERTp/WinA-yCD, d) CMVp/WinA-yCD, and e) pcDNA3.1neo and pcDNA3.1zeo mock vectors.) pcDNA3.1neo and pcDNA3.1zeo mock vectors. 48 hrs after transfection, cells were grown in medium containing 400 μg/ml of G418 and 400 μg/ml of Zeocin™ in order to select for stable cell lines. Selection medium was changed every 2 days. After 7 to 21 days, stable cell lines were trypsinized and transferred to 5 cm Petri dishes. Subsequently cells were transferred to 10 cm Petri dishes and grown in medium containing 200 μg/ml of G418 and 200 μg/ml of Zeocin™. These stable cell lines were allowed to reach approximately 70% confluency and frozen for later use.

2.12 5-FC Assay in Mammalian Cells and MTT Assay

1 $X10^4$ cells were seeded in 96-well plate with culture medium containing 200 μg/ml of G418 and 200 μg/ml of Zeocin™. After 24 hrs of incubation, the medium was changed for medium containing 0, 100 or 1000 μg/ml 5-FC and 200 μg/ml of G418 and 200 μg/ml of Zeocin™. Media were changed once every two days. After six days of treatment with 5-FC, cell viability was assayed by measuring the activity of dehydrogenase enzymes presence in metabolically active cells using the Colorimetric (MTT) assay for cell survival and proliferation kit purchased from Chemicon (Temecula, CA). Briefly, 10 μl of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrasodium bromide (MTT) in PBS, was added to each well of the 96-well plate and incubated for 3 hrs at 37°C. After the incubation time, 100 μl of solubilizing solution was added to each well in order to lyse cells and solubilize the product. Conversion of the yellow MTT substrate to a blue product was measure at 570 nm and 630 nm (Molecular Device Spectramax 190). The quantity of blue product produced is calculated by substracting the optical density (OD) value at 570 nm minus the OD at 630 nm. The percentage of relative growth inhibition is calculated with the following formula:

$= [(OD_{570nm-600nm}$ untreated - $OD_{570nm-600nm}$ 5-FC treated)/ $OD_{570nm-600nm}$ untreated] * 100

CHAPTER3: RESULTS

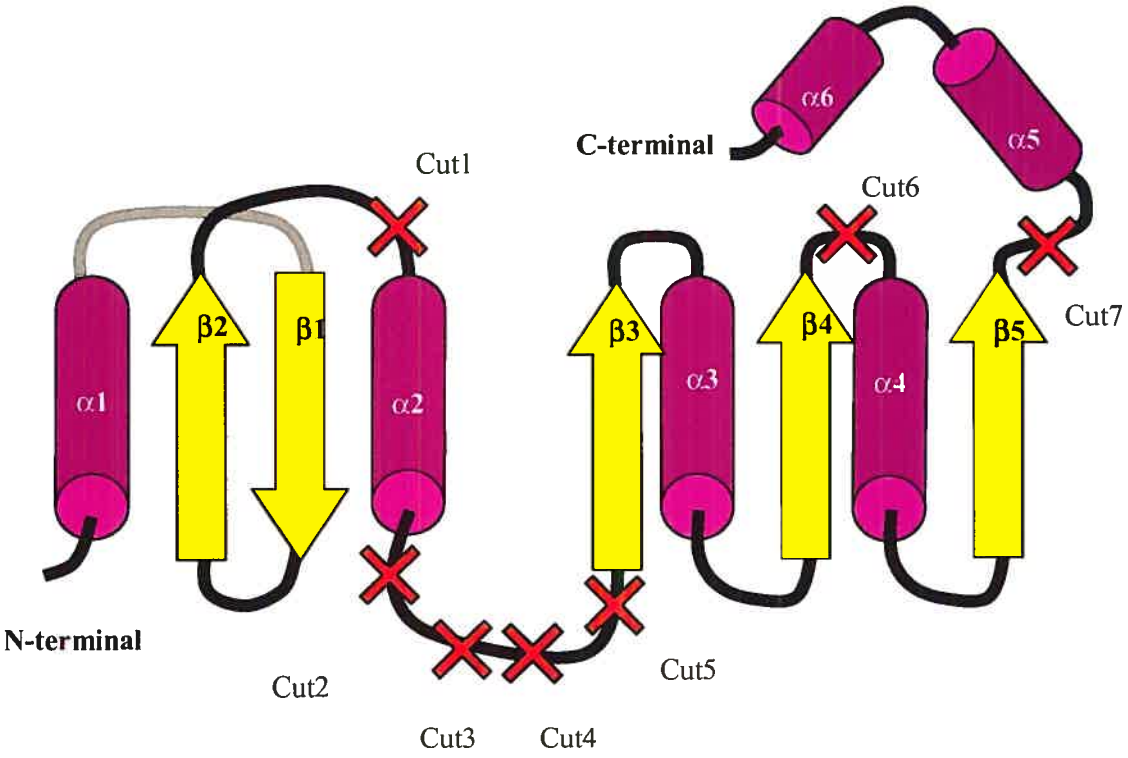
3.1 Development of yCD PCA in Yeast

We have chosen to develop a Positive/Negative Selection PCA in yeast for mainly two reasons. First, it is both time- and cost-efficient to use yeast for the developmental stage of our yCD PCA since no sophisticated equipment or material are required. A simple vector system (p413Gal1 and p415Gal1) with auxotrophic markers (Mumberg, Muller et al. 1994) and basic transformation protocol (Knop, Siegers et al. 1999) allowed us to study the effect of our fusion gene in yeast cells. It was not necessary to establish stable cell lines as would have been for mammalian cells in order to maintain stable expression of the recombinant genes. In addition, we took advantage of the haploid/diploid life cycle of yeast in order to introduce one fragment of our yCD PCA in *MAT***a** mating type and the other fragment in *MAT*α mating type and then mated the *MAT***a** and *MAT*α to generate diploid cells carrying the two fusion genes. The second reason was that we hoped also to establish a negative selection PCA in yeast for future application in screening projects. *In vivo* negative selection systems have been established to screen for bioactive molecules in bacteria and in yeast based on the concept of a reverse two hybrid assay (Licitra and Liu 1996; Horswill, Savinov et al. 2004) . The yCD PCA could be used for similar applications and provide a more direct method of signal output since yCD PCA does not involve the transcriptional regulatory system of a reporter gene (Michnick 2004).

3.1.1 Design and Construction of yCD PCA

Topological analysis of yCD structure revealed several loop regions of the protein that could serve as sites for fragmentation of the protein. Only one large loop region, composed of 10 amino acid residues, located between α2-helix and β3-strand (Figure 7), was identified from the yCD structure (Protein Data Bank file: 1UAQ). We considered this loop region as a good cut site for yCD fragmentation since it dissects the active site of the enzyme, glutamine-64 (E-64), from the zinc ion-binding signature motif (C-91-X-X-C-94) of yCD which coordinates the substrate for the deamination process. Hence, yCD fragments generated from cuts in this region were predicted to be unlikely to possess full enzymatic activity alone. We therefore chose four cut sites in this region (Figure 7). In addition, we have included other loop regions for yCD fragmentation in this study. Other cut sites were located in the β2-α2, β4-α4, and β5-α5 loop regions. In brief, the seven cut

Figure 7. yCD cut sites for PCA development. All cut sites are in loop regions of the protein. Cut site 1 is between K-56 and G-57. Cut site 2 is between R-73 and L-74. Cut site 3 is between G-76 and K-77. Cut site 4 is between K-77 and V-78. Cut site 5 is between K-80 and D-81. Cut site 6 is between N-111 and V112. Cut site 7 is between V-132 and D-133.



Legend for Cut Sites:

Cut1: K-56/G-57
Cut2: R-73/L-74
Cut3: G-76/K-77
Cut4: K-77/V-78
Cut5: K-80/D-81
Cut6: N-111/V-112
Cut7: V-132/D-133

sites were: cut site 1 (between amino acid 56-57), cut site 2 (between amino acid 73-74), cut site 3 (between amino acid 76-77), cut site 4 (between amino acid 77-78), cut site 5 (between amino acid 80-81), cut site 6 (between amino acid 111-112), and cut site 7 (between amino acid 132-133). This enzyme is very small and almost every α-helix and β-strand interacts with the substrate. Fragmentation of yCD between amino acid residues that serve to make contact with the substrate and hold it in place represents a potential cut site for yCD PCA since this could prevent the enzyme from catalyzing the deamination reaction. We avoided cutting in the middle of secondary structure of α-helices and β-strands since this could affect important structural elements for refolding of the enzyme.

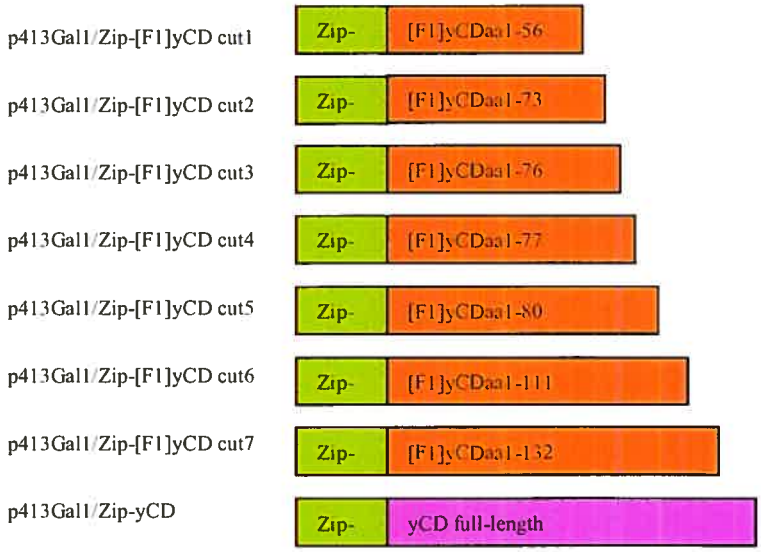
Constructions of fusion genes, used to determine yCD PCA activity, were done in p413Gal1 or p415Gal1 vectors and are listed in Figure 8. The fusions genes were cloned downstream of the Gal1 promoter which gives a high level of expression in yeast when yeast cells are grown in medium with galactose as an inducer and no glucose (Ronicke, Graulich et al. 1997). Fragments of yCD or full length yCD were fused at the C-terminus of the GCN4 leucine zipper. A linker region composed of 15 or 12 amino acids [$(GGGGS)_3$ or $(GGGGS)_2GG$] was inserted between the zipper and yCD fragments or yCD. All fusion genes presented in this work were generated with the same strategy.

3.1.2 Fusion Protein Expression

Expression of the fusion proteins was verified by Western blot using polyclonal anti-yCD antibody (Biogenesis). Detection of yeast 3-phosphoglycerate kinase (PGK), a ubiquitously expressed protein in yeast, was used as a loading control to indicate that a similar amount of protein had been loaded in each well of the acrylamide gel. In comparison to the mock samples (cells transformed with the mock p413Gal1 or p415Gal1 vectors) most yCD-fusion proteins were detected. Zip-[F2]yCDcut7 was expressed at a very low level (Figure 9) since it could only be detected on the same western blot at a longer exposure (data not shown). Unfortunately, the polyclonal anti-yCD antibody non-specifically detected other yeast cellular proteins (20 Kda and 24 Kda in size) in addition to yCD. The expression of Zip-[F1]yCD cut6, Zip-[F1]yCD cut7, and Zip-[F2]yCD cut1 (Figure 9) fusion proteins could not be confirmed by western blot analysis using the

Figure 8. Recombinant genes generated from yCD or yCD fragments fused to GCN4 zipper sequence. A) yCD Fragment 1 [F1] and yCD full-length enzyme, generated from the different cut sites, were fused to GCN4 zipper forming sequences and cloned into p413Gal 1 plasmid. B) yCD Fragment 2 [F2] and yCD full-length enzyme, generated from the different cut sites, were fused to GCN4 Zipper forming sequences and cloned into p415Gal 1 plasmid.

A) Fusion of GCN4 Zipper with yCD and yCD fragments



B) Fusion of GCN4 Zipper with yCD and yCD fragments

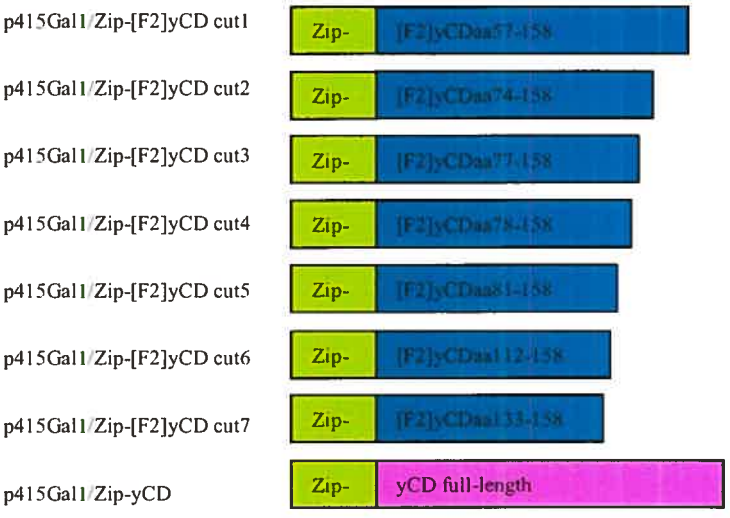
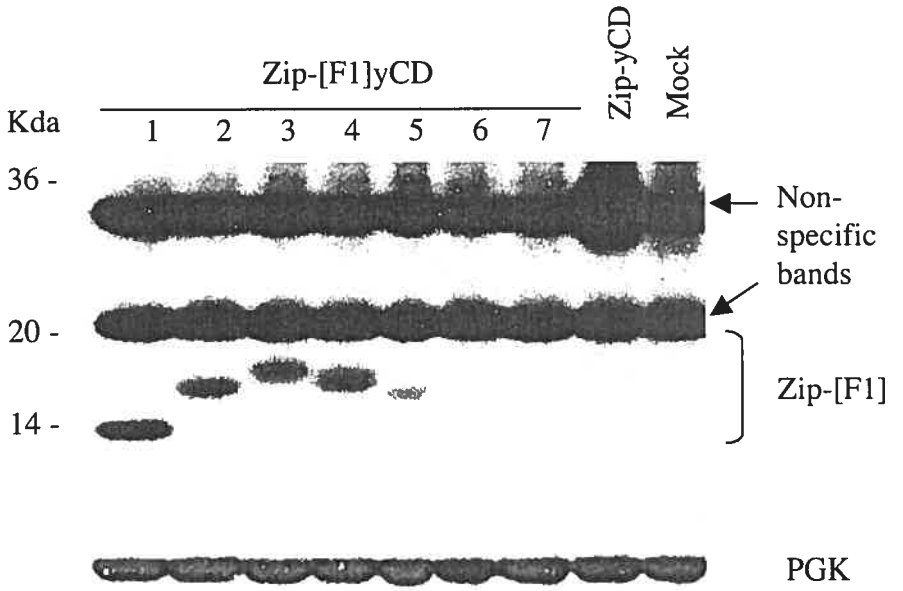
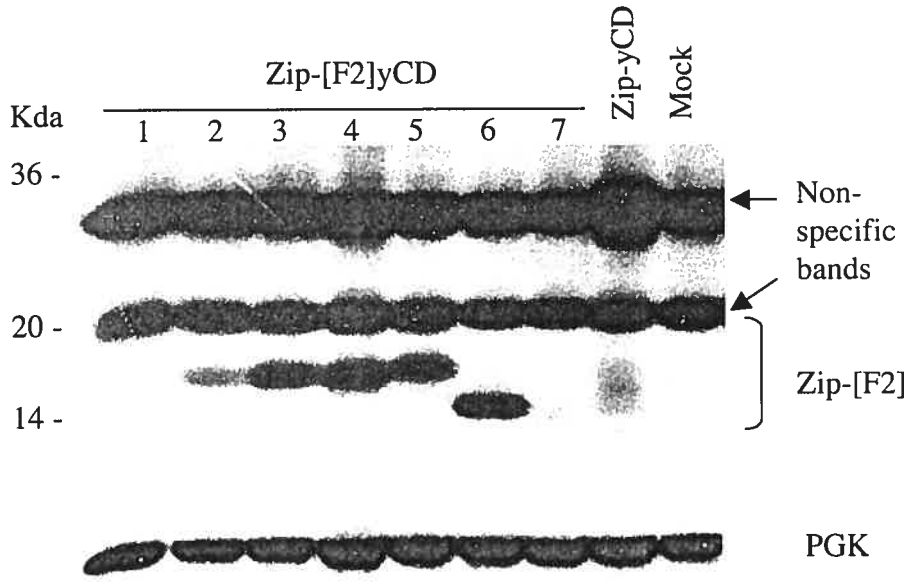


Figure 9. yCD PCA expression detection normalized to PGK expression. A) Zip-[F1]yCD (lane 1 to 7), Zip-yCD full-length enzyme (lane 8), and mock vector (lane 9) are probed with anti-yCD antibody. The membrane is then reprobed for anti-PGK as a loading control. B) Zip-[F2]yCD (lane 1 to 7), Zip-yCD full-length enzyme (lane 8), and mock vector (lane 9) are probed with anti-yCD antibody. The membrane is then reprobed for anti-PGK as a loading control.

A)


Zip-[F1]yCD
Zip-yCD
Mock
Kda
1
2
3
4
5
6
7
36 -
20 -
14 -
Non-
specific
bands
Zip-[F1]
PGK

B)


Zip-[F2]yCD
Zip-yCD
Mock
Kda
1
2
3
4
5
6
7
36 -
20 -
14 -
Non-
specific
bands
Zip-[F2]
PGK

polyclonal anti-yCD antibody since their expected molecular weights are approximately the same as the nonspecific bands however, activity of yCD PCA involving these three fusion proteins could be detected (see section 3.2), suggesting that they were expressed.

3.1.3 yCD PCA Activity in Liquid Medium

To assay for yCD PCA activity, we performed a death assay in liquid medium to provide a quantitative comparison of the activity of yCD PCA generated from different cut sites to that of full length yCD. As mentioned in the introduction, the yCD knockout yeast cannot deaminate 5-FC, a relatively non-toxic prodrug, to 5-FU, a toxic anti-metabolite, and are therefore resistant to high concentration of 5-FC (10^{-2} M) (Erbs, Exinger et al. 1997). Thus, the yCD PCA activity could be determined as a function of reconstituted yCD activity, which could convert 5-FC to 5-FU, resulting in inhibition of cell growth. The death assay consisted of measuring the percent cell growth inhibition of yeast in the presence of 100 μg/ml 5-FC (aproximately 10^{-4} M 5-FC). This concentration of 5-FC was determined to inhibit the growth of cells carrying a wild type copy of *FCY1* gene (data not shown). Only cells with yCD full length or reconstituted yCD fragments were sensitive to the toxic effect of the prodrug 5-FC. Two different culture conditions have been tested in this experiment, using different amounts of cells (Figure 10). According to both culture conditions, the activity of yCD PCA, determined as 5-FC sensitivity, was found for the cut site 3 and 4. Cut site 4 (between K-77/V-78) seemed to show higher activity than cut site 3 (between G-76/K-77) under both conditions. Cells transformed with Zip-yCD full-length constructs were highly sensitive to 100 μg/ml 5-FC whereas cells transformed with mock vectors were relatively non-sensitive to 100 μg/ml 5-FC.

3.1.4 Death Assay on Solid Media Using yCD PCA at Cut Site 4

Selection on solid medium is an important method for isolation of clones in screening projects. A population of different cells from a liquid culture can be selected by plating on solid selection medium in order to obtain individual clones. The clones could later be expanded to clonal populations. It was therefore important for us to determine selection conditions on solid medium. Initially, we directly plated yeast cells carrying yCD PCA on 5-FC selection plates however; this method of selection was not very efficient for

Figure 10. yCD PCA activity as measured by sensitivity to 5-FC in liquid medium. Diploid cells carrying Zip-[F1]yCD and Zip-[F2]yCD generated from cut site 1 to cut site 7, Zip-yCD full-length enzyme and mock empty vectors were assayed for yCD PCA activity in SDC-met-lys-his-leu +2% Raffinose + 2% Galactose + 200 μg/ml of G418 and 100 μg/ml of 5-FC. Two different growth conditions were assayed. ■ 5 X 10^3 cells were transferred to 250μl of selection medium and OD_{600} was taken after 24 hrs incubation. ❑ 5 X 10^2 cells were transferred to 250μl of selection medium and OD_{600} was taken after 24 hrs incubation. The relative percentage cell growth inhibition was calculated by comparing cells treated and untreated with 100 μg /ml of 5-FC.

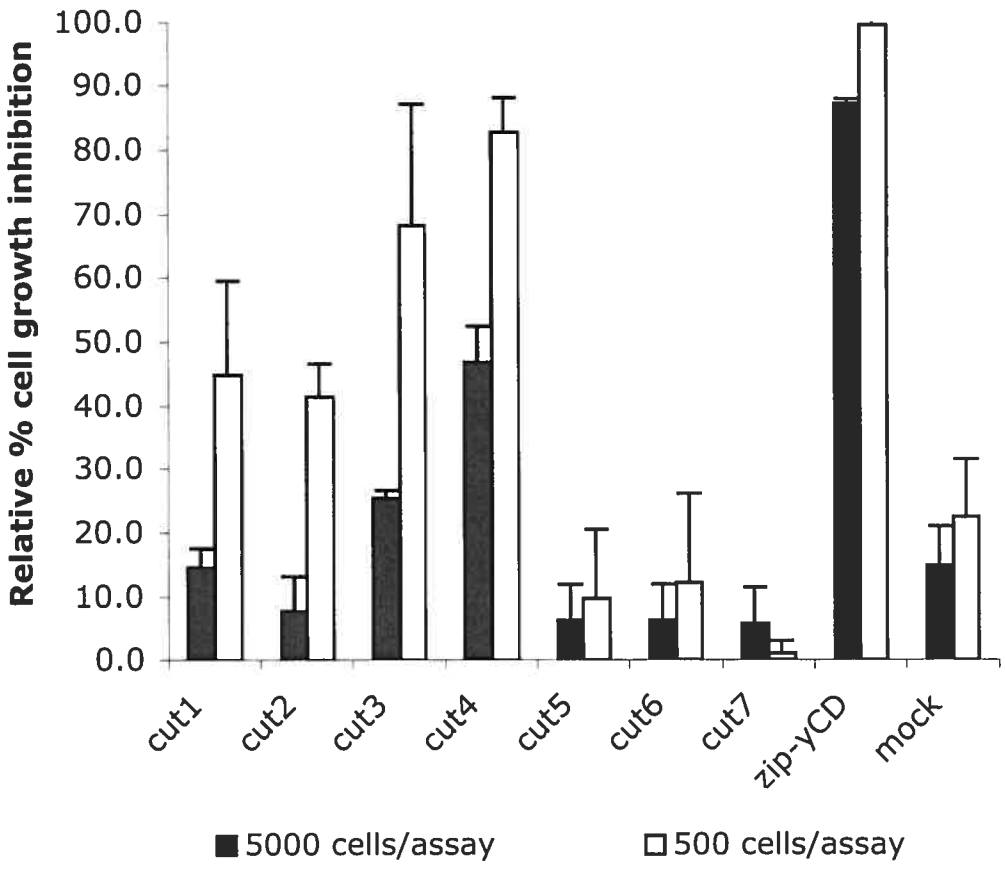
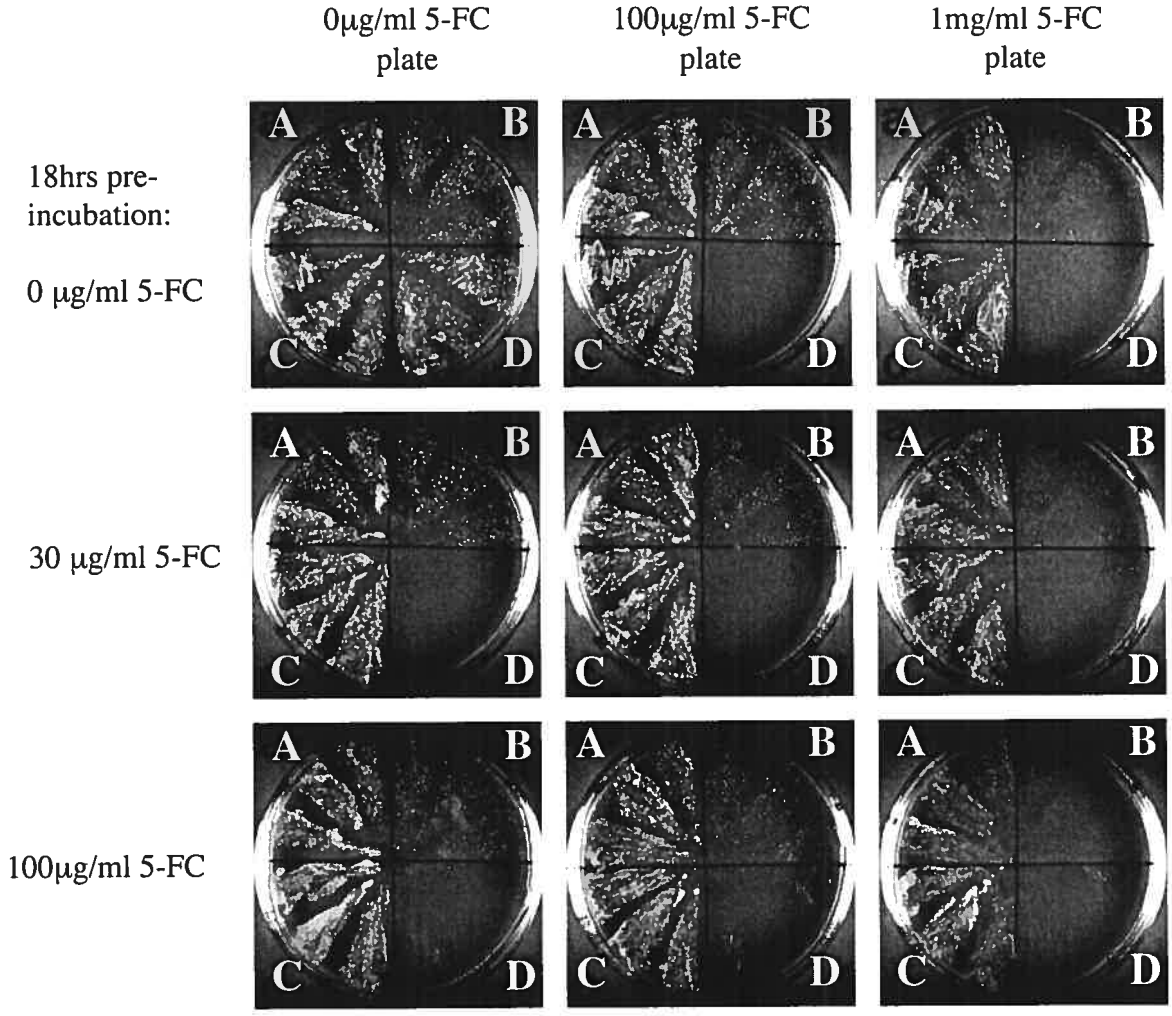

Relative % cell growth inhibition
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
cut1
cut2
cut3
cut4
cut5
cut6
cut7
zip-yCD
mock
5000 cells/assay
500 cells/assay

Figure 11. yCD PCA and 5-FC sensitivity on solid media (using fragments of cut site 4). Samples were pre-incubated for 18 hrs with 0, 30, or 100 μg/ml 5-FC in liquid selection medium. After pre-incubation time, 10 μl of each sample were plated on solid selection medium containing 0, 100 and 1000 μg/ml of 5-FC. Each Petri dish is subdivided into four quadrants and different samples were plated in each quadrant (quadrant **A:** Ras-[F1]yCD cut4 +Zip-[F2]yCD cut4, quadrant **B**: Zip-[F1]yCD cut4 + Zip-[F2]yCD cut4, quadrant **C**: p413Gal1 + p415Gal1 mock vectors, quadrant **D**: Zip-yCD).


0μg/ml 5-FC plate
100μg/ml 5-FC plate
1mg/ml 5-FC plate
18hrs pre-incubation:
0 μg/ml 5-FC
30 μg/ml 5-FC
100μg/ml 5-FC
A
B
C
D

the death selection assay since cells carrying Zip-[F1]yCD cut4 and Zip-[F2]yCD cut4 could still grow (data shown as control samples in Figure 11 top row). Subsequently, we attempted to optimize the selection conditions for yCD PCA on solid media by pre-incubating cells with various amount of 5-FC in order to increase the selection stringency. Samples were pre-incubated for 18 hrs with 0, 30, or 100 μg/ml 5-FC in liquid selection medium and plated on solid selection medium containing 0, 100 or 1000 μg/ml of 5-FC (Figure 11).

With no prior pre-incubation in 5-FC, cells carrying Zip-yCD fusion protein (displayed in quadrant **D** of each Petri) did not grow on medium containing 100 and 1000 μg/ml of 5-FC. Cells carrying Zip-[F1]yCD cut4 and Zip-[F2]yCD cut4 (quadrant **B**) showed significantly reduced growth on 1000 μg/ml of 5-FC plate in comparison to the following negative controls: cells carrying non-interacting protein pair, Ras-[F1]yCD cut4 and Zip-[F2]yCD cut4 (quadrant **A**) and mock vectors (quadrant **C**). Cells carrying mock vectors (quadrant **C**) and non-interacting protein pair (quadrant **A**) grew on all selection plates although less colonies were observed on 1000 μg/ml of 5-FC plate due to the general toxicity effect of 5-FC when used at such a high concentration.

With prior pre-incubation in 30 or 100 μg/ml of 5-FC, cells carrying Zip-yCD fusion protein could not form colonies when plated on medium containing 0, 100, or 1000 μg/ml 5-FC. Cells carrying the Zip-[F1]yCD cut4 and Zip-[F2]yCD cut4 showed reduced growth on 100 and 1000 μg/ml of 5-FC plate in comparison to the non-interacting proteins and mock control. An 18 hrs pre-incubation with 100 μg/ml of 5-FC and plating on solid medium containing 1000 μg/ml of 5-FC seemed to give best results for inhibiting growth of cells carrying the GCN4 zipper homodimer fused to yCD fragments.

3.2 **Optimizing yCD PCA**

PCA, carrying overlapping amino acid residues of the reporter, have been found to generate an increase in PCA activity (Wehrman, Kleaveland et al. 2002). Since yCD has been fragmented at seven cut sites for determining yCD PCA activity, shuffling different fragment 1 versus fragment 2 can generate yCD PCA with overlapping sequences. Testing

for the PCA activity of these combinations can be a simple strategy to screen for a new yCD PCA that have better PCA activity.

3.2.1 Fragment Swapping of yCD PCA Activity in Liquid Medium

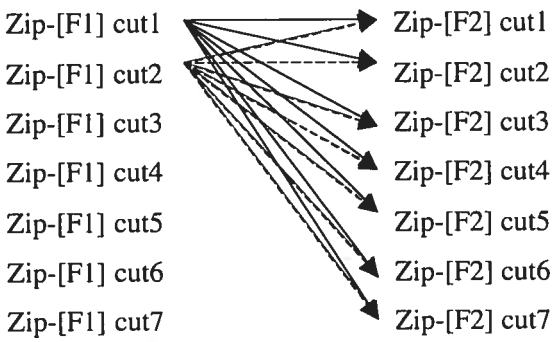
We attempted to increase yCD PCA activity by combining fragment one of yCD ([F1]yCD) of the seven different cut sites with fragment two of yCD ([F2]yCD) of the seven different cut sites. For example, we mated each *MAT***a** expressing Zip-[F1]yCD cut1 with seven *MAT*α expressing different Zip-[F2]yCD cut1 through cut 7 (Figure 12 A). This process was then repeated for [F1]yCD of the other six remaining cut sites. We tested all 49 different combinations and have found more yCD PCA activity in other [F1]yCD/[F2]yCD combinations (Figure 12 B) than the original findings determined for cut site 3 and 4 (Figure 10). There were other combinations of yCD PCA (marked with + or ++ in Figure 12 B) that gave some yCD PCA activity however; they were equal to (noted as ++) or less than (noted as +) the original yCD PCA activity at cut site 4. All yCD PCA containing two overlapping α2 helices (Figure 7) gave higher yCD PCA activity in terms of sensitivity in the 5-FC death assay. The presence of these α2 helices could stabilize the full-length enzyme in a monomeric form and thus enhance yCD PCA activity. Explanation of this observation is further presented in the Discussion Section.

3.2.2 Comparison of yCD PCA Activity on Solid Media

Selection on solid medium is important for isolation of clones since we would like to use this PCA for screening genetic library of bioactive molecules using cyclic peptides (Scott, Abel-Santos et al. 1999). We tested various conditions for solid media selection with 5-FC pre-incubation (Figure 13). Selection conditions with an 18 hrs pre-incubation with 100 μg/ml 5-FC and plating on selection plates containing 1000 μg/ml 5-FC seems to give good results. The negative control (diploid cells carrying Ras-[F1]yCD cut 4 and Zip-[F2]yCD cut 4 plated on quadrant **A**) and the mock control (diploid cells carrying p413Gal1 and p415Gal1 plated on quadrant **C**) grew on solid medium selection containing 1000 μg/ml 5-FC compared to cells containing Zip-[F1]yCD cut4 and Zip-[F2]yCD cut4 which grew very little or cells carrying Zip-yCD full length which did not grow at all. Ras-[F1]yCD cut 4 and Zip-[F2]yCD cut 4 were used as negative control since Ras does not interact with GCN4 leucine zipper.

Figure 12. Fragment swapping matrix of yCD-PCA activity in liquid medium. A) *MAT***a** cells carrying Zip-[F1]yCD cut1 were mated with *MAT*α cells carrying Zip-[F2]yCD of different cut sites (cut1 to cut7). This process was repeated with *MAT***a** cells carrying Zip-[F1]yCD cut 2 to Zip-[F1]yCD cut7. B) 5 X 10^3 cells were assayed for their sensitivity to 100 μg/ml 5-FC and OD_{600} was taken after 24 hrs of incubation. Results are qualitatively represented on the matrix: +++, highest yCD PCA activity; ++, moderate yCD PCA activity; +, low yCD PCA activity.

A



B

	Zip-[F2] cut1	Zip-[F2] cut2	Zip-[F2] cut3	Zip-[F2] cut4	Zip-[F2] cut5	Zip-[F2] cut6	Zip-[F2] cut7
Zip-[F1] cut1	+				+	+	
Zip-[F1] cut2	+++				+		
Zip-[F1] cut3	+++		+		+		+
Zip-[F1] cut4	+++			++	+		
Zip-[F1] cut5	++		+	+		+	
Zip-[F1] cut6	++						
Zip-[F1] cut7			+				

Figure 13. Comparison of yCD PCA activity using 5-FC sensitivity assay on solid selection media. Samples were pre-incubated for 18 hrs with 100 μg/ml 5-FC in liquid selection medium. After pre-incubation time, 10 μl of each sample was plated on solid selection medium containing 0, 100 or 1000 μg/ml of 5-FC.

5-FC concentration in μg/ml

0 100 1000

a) Zip-[F1]yCD cut1
Zip-[F2]yCD cut1

b) Zip-[F1]yCD cut1
Zip-[F2]yCD cut4

c) Zip-[F1]yCD cut4
Zip-[F2]yCD cut1

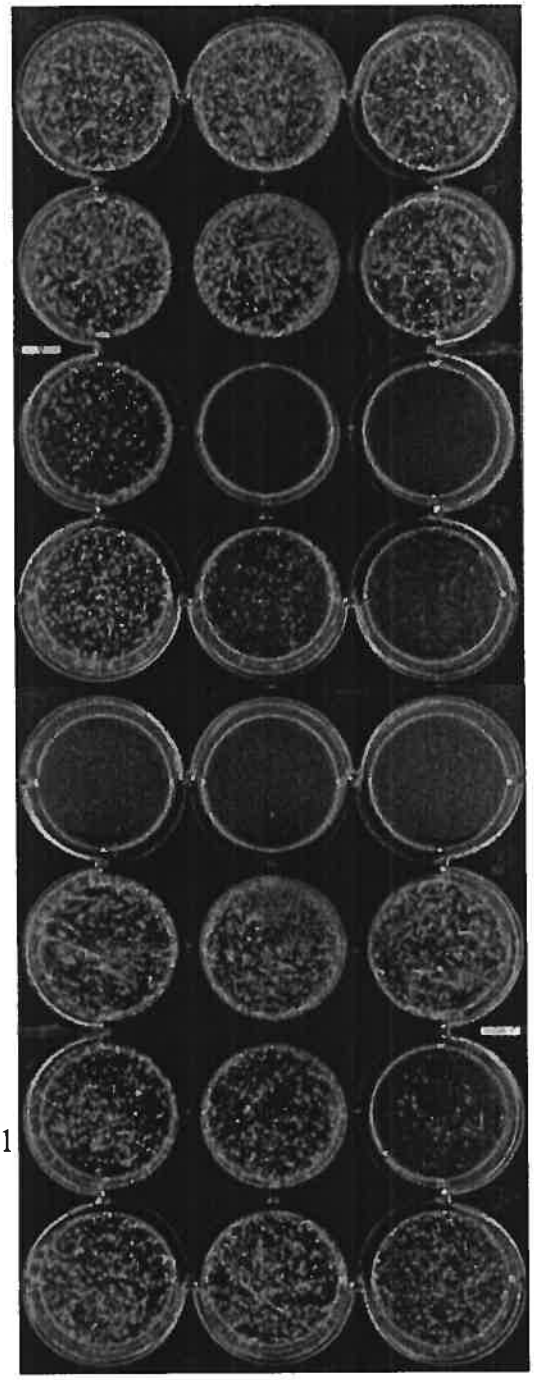
d) Zip-[F1]yCD cut4
Zip-[F2]yCD cut4

e) Zip-yCD
p415 Gal1

f) p413 Gal1
p415 Gal1

g) Ras-[F1]yCD cut4
RBD-[F2]yCD cut1

h) Ras-[F1]yCD cut4
Zip-[F2]yCD cut1



Using the same selection condition, we had compared the activity of yCD PCA at cut site 4 (Zip-[F1]yCD cut4 and Zip-[F2]yCD cut4) with yCD PCA at cut site 1 (Zip - [F1]yCD cut1 and Zip-[F2]yCD cut1), and yCD PCA containing overlapping α2 helices (Zip-[F1]yCD cut4 and Zip-[F2]yCD cut1) on solid selection medium. Since the overlapping α2 helices yCD PCA seems to work better in liquid assay, we suspected that yCD PCA with overlapping α2 helices could also work better than the other combinations on solid medium selection as well. Results from the solid medium selection showed that cells carrying Zip-[F1]yCD cut4 and Zip-[F2]yCD cut1 did not grow with 100 or 1000 μg/ml 5-FC (Figure 13c). Cell carrying Zip-[F1]yCD cut4 and Zip-[F2]yCD cut4 showed significantly reduced growth however, some colonies can still be observed on selection medium containing 1000 μg/ml 5-FC (Figure 13d). Cells carrying Zip-[F1]yCD cut 1 and Zip-[F2]yCD cut1 (Figure 13a), found to have low yCD PCA activity when tested using the liquid selection assay (Figure 10), did not show reduced growth on solid medium supplemented with high 5-FC concentration (1000 μg/ml 5-FC). Cells carrying Zip-[F1]yCD cut 1 and Zip-[F2]yCD cut4, in which 21 amino acids of yCD were absent, were also non-sensitive to 5-FC (Figure 13b). The controls of this experiment were cells carrying Zip-yCD full-length and empty vectors. As expected, cells carrying Zip-yCD full-length were sensitive to 5-FC (Figure 13e). Cells did not grow when plated on medium without 5-FC due to the fact that they were pre-incubated with 5-FC for 18 hrs. This result is consistent with results observed when determining solid medium selection conditions for yCD PCA (Figure 11). Cells carrying mock vectors were resistant to high concentration of 5-FC.

In addition, we tested the overlapping α2 helices yCD PCA using another interacting protein partners rather than the interacting homodimeric GCN4 Zipper. We chose Ras and Ras Binding Domain of Raf-1 (RBD) as the interacting partners. The interaction of Ras and RBD has been detected using the mDHFR (Pelletier, Campbell-Valois et al. 1998) and beta-lactamase PCA (Pelletier, Campbell-Valois et al. 1998). Ras was fused to fragment 1 of yCD and RBD was fused to fragment 2 of yCD (Ras-[F1]yCD cut4 and RBD-[F2]yCD cut1). Cells carrying these recombinant proteins were plated on

the solid selection medium. Although not all cells were killed, a difference in cell growth inhibition can be observed (Figure 13g) in comparison to the negative control, cells carrying Ras-[F1]yCD cut4 and Zip-[F2]yCD cut1, which were non-sensitive to 5-FC (Figure 13h). We have also tested Ras-[F1]yCD cut4 and RBD-[F2]yCD cut4, which did not contain the overlapping α2 helices. However, cells were not sensitive to 5-FC (data not shown).

3.2.3 Alternative Survival Assay on Solid Media

yCD PCA activity can be selected using a survival assay in a yeast strain in which the pyrimidine *de novo* pathway is disrupted (eg. *URA*3 gene deletion strain) and uracil is absent in the culture medium. Under such conditions, only cells that can deaminate cytosine to uracil using yCD can grow. We tested yCD PCA using this cytosine survival assay and found that cells transformed with yCD PCA (Zip-[F1]yCD cut4 and Zip-[F2]yCD cut1) and Zip-yCD grew after three days of incubation at 30°C with cytosine supplementation of 1000 μg/ml and 100 μg/ml respectively (Figure 14A). No colonies were observed for yCD PCA Zip-[F1]yCD cut4 and Zip-[F2]yCD cut4 (Figure 14A (b)) or any of the negative controls: Ras-[F1]yCD cut4 and Zip-[F1]yCD cut4, Ras-[F1]yCD cut4 and Zip-[F1]yCD cut1) (data not shown). When the selection assay was prolonged to six days incubation time, colonies were detected for yCD PCA (Zip-[F1]yCD cut4 and Zip-[F2]yCD cut4) with 1000 μg/ml cytosine supplementation (Figure 14B (b)) and colonies were observed for yCD PCA Zip-[F1]yCD cut4 and Zip-[F2]yCD cut1 with 100 μg/ml cytosine supplementation.

3.3 Application of yCD PCA in cancer gene therapy

Previous studies have shown that yCD can be used in combination with tissue specific promoters and 5-FC to specifically ablate tumor cells (Nyati, Sreekumar et al. 2002; Song, Kim et al. 2003). yCD PCA can also be used as a death assay to kill a population of cells by converting 5-FC to 5-FU. We applied this death assay under the expression of two tissue specific promoters in order to specifically drive the expression of yCD PCA and kill cancer cells when treated with 5-FC. Using yCD PCA under the control

Figure 14. yCD PCA activity using the survival assay on solid selection media. Cells were grown overnight in 2% Raffinose and induced for 6 hrs in 2% Raffinase + 2% Galactose. After the induction time, 10 µl of cells were plated on selection medium that does not contain uracil but is supplemented with 0, 100 or 1000 µg/ml of cytosine. A) Pictures were taken after 3 days of incubation at 30°C. B) Pictures were taken after 6 days of incubation at 30°C.

A)

Cytosine concentration in μg/ml

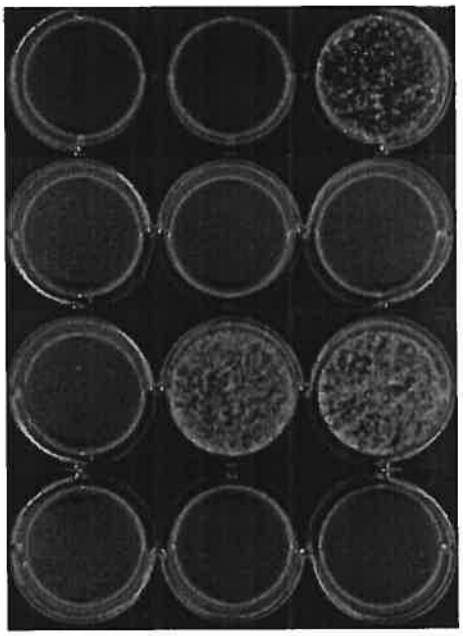
0 100 1000

a) Zip-[F1]yCD cut4
Zip-[F2]yCD cut1

b) Zip-[F1]yCD cut4
Zip-[F2]yCD cut4

c) Zip-yCD
p415 Gal1

d) p413 Gal1
p415 Gal1



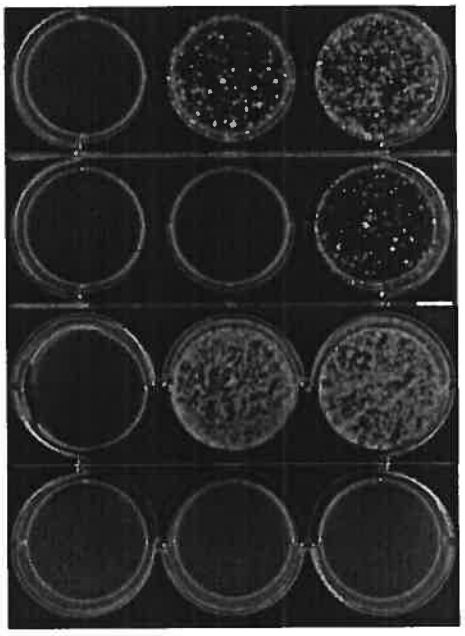
B)

a) Zip-[F1]yCD cut4
Zip-[F2]yCD cut1

b) Zip-[F1]yCD cut4
Zip-[F2]yCD cut4

c) Zip-yCD
p415 Gal1

d) p413 Gal1
p415 Gal1



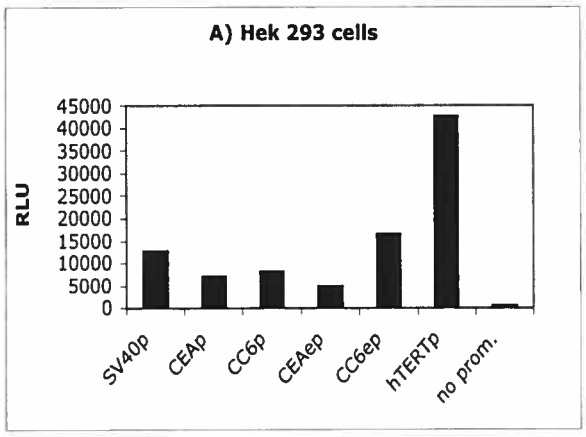
of two tissue specific promoters could further increase the specificity of targeting the yCD/5-FC deamination process to tumor cells.

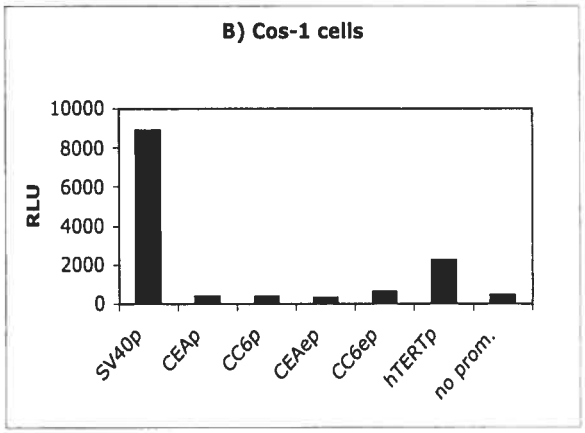
3.3.1 Re-Characterization of Tissue Specific Promoters

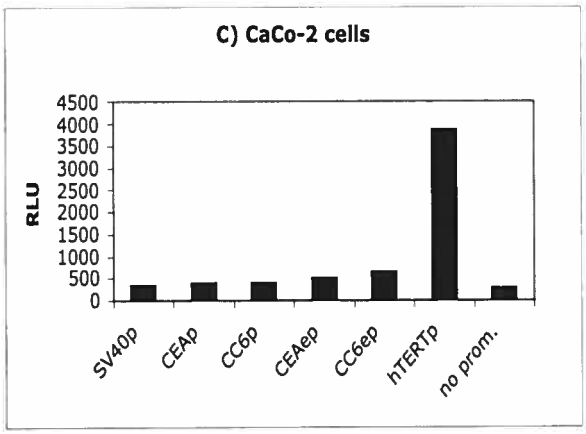
We re-characterized tissue specific promoters previously described by other groups (Hauck and Stanners 1995; Koops, Thompson et al. 1998; Nyati, Sreekumar et al. 2002). We PCR amplified promoter sequences of CEA, CC6 and hTERT genes from genomic DNA of LoVo cells and subcloned them upstream of the luciferase gene in the pGL3 vector in order to verify if the PCR products have promoter activity. In total, we generated five vectors containing the following promoter regions: CEA promoter from -407 to –43 bp (CEAp), CEA promoter-enhancer from -6.1 to –4 kb and -407 to –43 bp (CEAep), CC6 promoter from -281 to –2 bp (CC6p), CC6 promoter-enhancer from -1.24 to –0.585 kb and -281 to –2 bp, and hTERT promoter from –204 to +56bp (hTERTp).

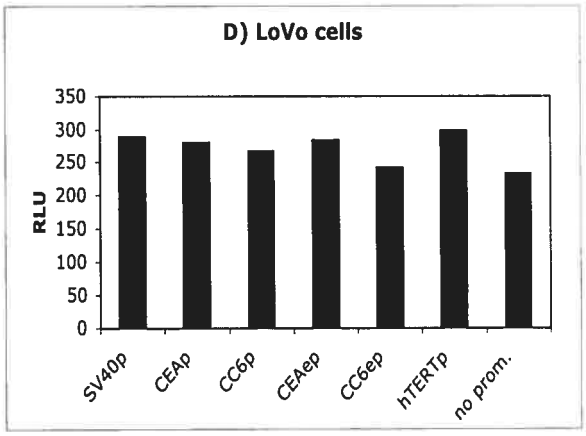
We compared the five promoters to the SV40 promoter in tumor and non-tumor cell lines by transient transfection. All promoter activity using firefly luciferase as a reporter protein were normalized to results of the R*enilla* luciferase activity under the CMV promoter. Four human colon cancer cell lines were used in this study: CaCo2, LoVo, LS180, and SW1222. In addition, we tested the promoters in human embryonic kidney (HEK 293) cells since HEK 293 cells are easy to maintain in culture and transfect. Moreover, HEK 293 cells can serve as a positive control for hTERTp since the hTERTp has been known to be highly expressed in these cells (Ritz, Kuhle et al. 2005). We used a fibroblast cell line as a negative control for the promoter study since CEA and CC6 were previously characterized to be expressed at a very low levels in fibroblast tissue (Eades-Perner, van der Putten et al. 1994; Chan and Stanners 2004). We attempted to use human fibroblast, MG-63 cells, in order to generate a closer comparison with the other human cell lines. However, the transfection efficiency of MG-63 cells using Fugene, Lipofectamine, or electroporation was very low (data not shown). Instead, the monkey fibroblast Cos-1 cell line was used as negative control since Cos-1 cells can easily be transfected.

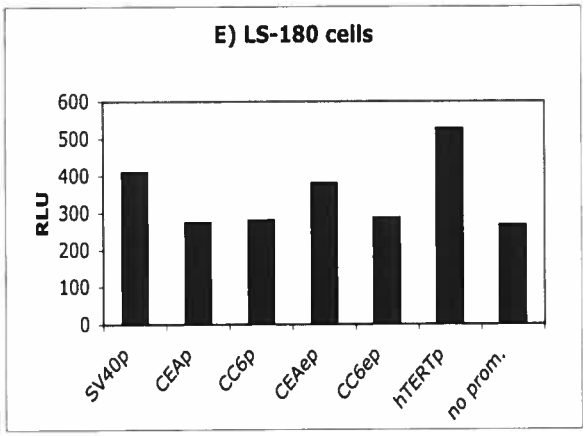
Figure 15. Comparison of tissue specific promoters activity in various cell lines using a transcriptional reporter assay. Previously characterized promoter sequence of CEA, CC6, and hTERT were amplified from genomic DNA of LoVo cells and sub-cloned into pGL3 vector. The plasmids were co-transfected with pRL-CMV into different cell lines and assayed for firefly and R*enilla* luciferase activity. Each panel displays normalized results of promoter activity in a particular cell line: A) HEK 293 cells, B) Cos-1 cells, C) CaCo-2 cells, D) LoVo cells, E) LS-180 cells, and F) SW1222 cells. Promoter Activity is expressed in Relative Luminescence Units (RLU).

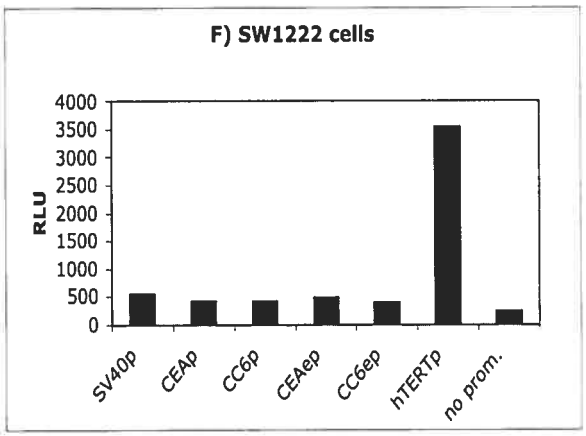

A) Hek 293 cells
RLU
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
SV40p
CEAp
CC6p
CEAep
CC6ep
hTERTp
no prom.


B) Cos-1 cells
RLU
10000
8000
6000
4000
2000
0
SV40p
CEAp
CC6p
CEAep
CC6ep
hTERTp
no prom.


C) CaCo-2 cells
RLU
4500
4000
3500
3000
2500
2000
1500
1000
500
0
SV40p
CEAp
CC6p
CEAep
CC6ep
hTERTp
no prom.


D) LoVo cells
RLU
350
300
250
200
150
100
50
0
SV40p
CEAp
CC6p
CEAep
CC6ep
hTERTp
no prom.


E) LS-180 cells
RLU
600
500
400
300
200
100
0
SV40p
CEAp
CC6p
CEAep
CC6ep
hTERTp
no prom.


F) SW1222 cells
RLU
4000
3500
3000
2500
2000
1500
1000
500
0
SV40p
CEAp
CC6p
CEAep
CC6ep
hTERTp
no prom.

We found that the activity of CEA, CC6, and hTERT promoters or promoter-enhancer sequences was not very active in monkey fibroblast Cos-1 cells (Figure 15 B) in comparison to the SV40 promoter. In contrast, these promoters were more active in HEK 293 cells (Figure 15 A). In general, the hTERT promoter was as active or up to thirty-three fold as active as the SV40 promoter in colon cancer cell lines (Figure 15). CEA and CC6 promoters were active at similar levels as SV40 promoter in CaCo-2 cells. We did not find a higher activity of CEA promoter-enhancer sequence as reported by Lawrence et al., 2002. There was also a slight increase in the promoter activity of CC6 promoter-enhancer when compared to CC6 promoter alone in colon cancer cell lines. From this promoter characterization experiment, we confirmed that the hTERT promoter sequence, isolated from genomic DNA of LoVo cells, can drive the expression of the luciferase gene to similar or higher expression levels as the SV40 viral promoter. These observations were consistent with previous promoter assay results (Hauck and Stanners 1995; Koops, Thompson et al. 1998; Song, Kim et al. 2003). In addition, since the CEA promoter has previously been reported to be highly active in cancer cell lines (Hauck and Stanners 1995; Koops, Thompson et al. 1998) and we have also found that it is highly expressed in CaCo-2 cells (Figure 15 C), we will use the hTERT and CEA promoter to drive the expression of two interacting protein partners fused to yCD fragments and target these cells for destruction using yCD PCA and 5-FC.

3.3.2 yCD PCA in Cancer Cell Lines

The general idea of using yCD PCA under tissue specific promoter is to specifically kill cancer cells which express both CEA and hTERT tumor marker genes, while sparing normal cells that may express only one of the two genes. We used tissue specific promoters of CEA and hTERT (Figure 15) to drive the expression of yCD PCA. In addition, in order to potentially further increase yCD PCA activity, we used WinZipA1 (WinA) and WinZipB1 (WinB) leucine zippers instead of the yeast GCN4 leucine zipper. WinA and WinB have been shown to heterodimerize with a dissociation constant of approximately 24 nM (Arndt, Pelletier et al. 2000) in comparison to GCN4 leucine zipper domain, which forms homodimers.

Table IV. List of Stable Cell-lines generated or in the progress of being generated for this study

	Original Cell-line	Constructs in pcDNA3.1neo	Constructs in pcDNA3.1zeo	Cell-line Establishment	5-FC Assay
1	HEK-293 cells	CMVp/WinA-F1yCDcut4	CMVp/WinB-F2yCDcut4	done	yes
2	HEK-293 cells	CMVp/WinA-F1yCDcut4	CMVp/WinB-F2yCDcut1	done	yes
3	HEK-293 cells	CMVp/WinA-F1yCDcut4	pcDNA3.1zeo	done	yes
4	HEK-293 cells	pcDNA3.1neo	CMV/WinB-F2yCDcut1	done	yes
5	HEK-293 cells	CMVp/WinA-yCD	pcDNA3.1zeo	done	yes
6	HEK-293 cells	pcDNA3.1neo	pcDNA3.1zeo	done	yes
7	HEK-293 cells	CEAp/WinA-F1yCDcut4	hTERTp/WinB-F2yCDcut4	done	yes
8	HEK-293 cells	CEAp/WinA-F1yCDcut4	hTERTp/WinB-F2yCDcut1	done	yes
9	HEK-293 cells	CEAp/WinA-F1yCDcut4	pcDNA3.1zeo	done	yes
10	HEK-293 cells	pcDNA3.1neo	hTERTp/WinB-F2yCDcut1	done	yes
11	HEK-293 cells	CEAp/WinA-yCD	pcDNA3.1zeo	done	yes
12	CaCo-2 cells	CMVp/WinA-F1yCDcut4	CMVp/WinB-F2yCDcut4	done	no
13	CaCo-2 cells	CMVp/WinA-F1yCDcut4	CMVp/WinB-F2yCDcut1	done	no
14	CaCo-2 cells	CMVp/WinA-F1yCDcut4	pcDNA3.1zeo	in progress	no
15	CaCo-2 cells	pcDNA3.1neo	CMV/WinB-F2yCDcut1	in progress	no
16	CaCo-2 cells	CMVp/WinA-yCD	pcDNA3.1zeo	done	no
17	CaCo-2 cells	pcDNA3.1neo	pcDNA3.1zeo	done	no
18	CaCo-2 cells	CEAp/WinA-F1yCDcut4	hTERTp/WinB-F2yCDcut4	done	no
19	CaCo-2 cells	CEAp/WinA-F1yCDcut4	hTERTp/WinB-F2yCDcut1	done	no
20	CaCo-2 cells	CEAp/WinA-F1yCDcut4	pcDNA3.1zeo	in progress	no
21	CaCo-2 cells	pcDNA3.1neo	hTERTp/WinB-F2yCDcut1	in progress	no
22	CaCo-2 cells	CEAp/WinA-yCD	pcDNA3.1zeo	done	no

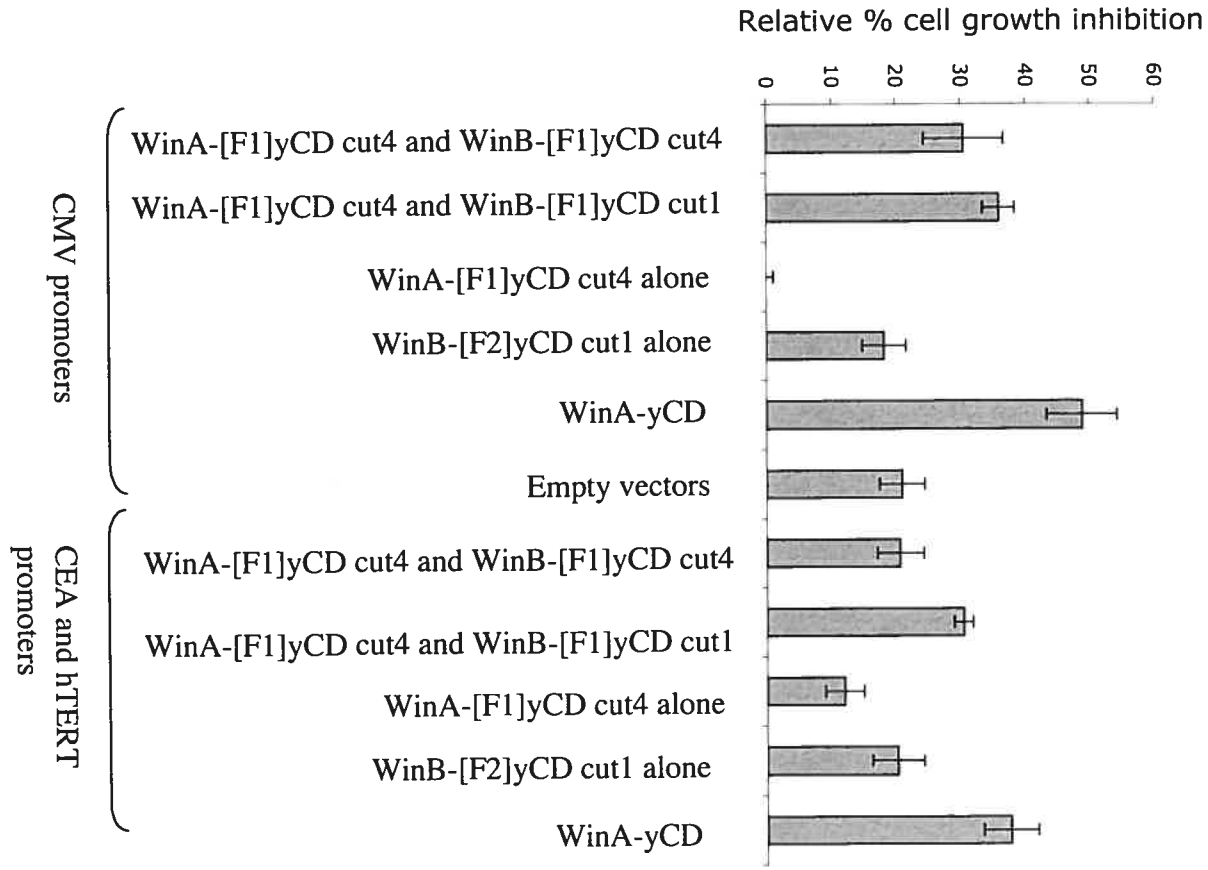
We fused WinA-[F1]yCD cut4 downstream of CEA and CMV promoters, WinB-[F2]yCD downstream of hTERT and CMV promoters, and WinA-yCD downstream of hTERT and CMV promoters. We used these vectors to establish stable cell lines of CaCo2 cells, a human colon cancer cell line, since the transfection efficiency was higher than other colon cancer cell lines (general observation from luciferase assay in Figure 15). In addition, we established stable HEK 293 cell lines since their transfection efficiency was also high and CEA and hTERT promoters were determined to be highly active.

In brief, we have generated or are in the process of generating the following stable cell lines in CaCo2 and HEK 293 cells (Table IV) and have tested some stable cell lines for their sensitivity to 5-FC. Since not all of the stable cell lines have been generated in the CaCo-2 colon cancer cell lines, we did not include results from the CaCo-2 stable cell lines in this thesis. The stable HEK 293 cell lines were tested by incubation in medium with and without 5-FC for 6 days. Since mammalian cells do not express cytosine deaminase, they are relatively non-sensitive to 5-FC. A final concentration of 1000 μg/ml 5-FC was used to study the effect of 5-FC (Figure 16). After the 6 days incubation, cell survival was quantitatively evaluated using the MTT assay, which measures the activity of dehydrogenase enzymes presence in metabolically active cells. Results are represented in percentage cell growth inhibition since cells carrying yCD or yCD PCA become sensitive to 5-FC, arrest their growth and undergo apoptosis (Fischer, Steffens et al. 2005). Approximately 20% cell growth inhibition was observed in HEK 293 stable transfectants carrying pcDNA3.1 neo and pcDNA3.1zeo mock vectors. Under the CMV promoter, HEK 293 stable cell lines carrying WinA and WinB fused to yCD PCA at cut site 4, had a 30.5 inhibition % of cell growth when treated with 1000 μg/ml of 5-FC. WinA and WinB fused to yCD PCA with the overlapping α2 helices showed about 36% cell growth inhibition. The negative controls, cells stably transfected with either WinA-[F1]yCD cut4 or WinB-[F2]yCD cut1 showed sensitivity that is below the mock controls. Cells carrying WinA-yCD showed approximately 48.9 % of cell growth inhibition.

Under CEA and hTERT tissue specific promoters, HEK 293 stable cell lines carrying WinA and WinB fused to yCD PCA at cut site 4, had 21 % of cell growth

inhibition when treated with 1000 μg/ml of 5-FC, which is approximately equivalent to the mock control. Therefore, no significant yCD PCA activity is observed for this cut site. WinA and WinB fused to yCD PCA with the overlapping α2 helices showed about 30.5% cell growth inhibition and negative controls, cells stably transfected with either WinA-[F1]yCD cut4 or WinB-[F2]yCD cut1, showed sensitivity that is below the mock controls. Cells carrying WinA-yCD under the CEA promoter showed around 37.8 % of cell growth inhibition.

Figure 16. yCD PCA activity in HEK 293 mammalian cells stable transfected with plasmids under CMV and tissue specific promoters expressing: WinA-[F1]yCD cut4 and WinB-[F2]yCD cut4, WinA-[F1]yCD cut4 and WinB-[F2]yCD cut1, WinA-[F1]yCD cut4 alone, WinB-[F2]yCD cut1 alone, WinA-yCD, and empty vectors. yCD PCA activity is expressed in relative % cell growth inhibition by comparing cells treated and untreated with 1000 μg/ml of 5-FC.


Relative % cell growth inhibition
0
10
20
30
40
50
60
CMV promoters
WinA-[F1]yCD cut4 and WinB-[F1]yCD cut4
WinA-[F1]yCD cut4 and WinB-[F1]yCD cut1
WinA-[F1]yCD cut4 alone
WinB-[F2]yCD cut1 alone
WinA-yCD
Empty vectors
CEA and hTERT promoters
WinA-[F1]yCD cut4 and WinB-[F1]yCD cut4
WinA-[F1]yCD cut4 and WinB-[F1]yCD cut1
WinA-[F1]yCD cut4 alone
WinB-[F2]yCD cut1 alone
WinA-yCD

CHAPTER4: DISCUSSION

4.1 Binary Positive and Negative yCD PCA

In this work, we established a new Binary Positive and Negative yCD PCA, which can be used for survival or death selection. Two factors permitted the dual selection strategies associated with this PCA. First, the death assay is possible because of the specific activity of yCD for catalyzing the deamination of 5-FC, a relatively non-toxic pro-drug to 5-FU, a toxic anti-metabolite, that can cause cell death (Zhang, Zhang et al. 2002; Fischer, Steffens et al. 2005). Second, the survival assay is achieved by genetically inactivating the yeast pyrimidine *de novo* synthesis pathway in order for yCD to become the essential regulatory enzyme in the cell. We focused on the negative selection aspect of this PCA since until now, no death PCA has been developed.

4.2 yCD PCA Activity

yCD is a relatively small enzyme of 17 KDa and the topology of the enzyme does not show many extended loop regions. We have fragmented the yCD enzyme in two fragments at seven different cut sites and fused each fragment to GCN4 leucine zipper domain. We established our assay to screen for restoration of yCD activity induced by GCN4 dimerization and yCD complementation using the 5-FC death assay and cytosine survival assay. From the seven cut sites, we found that yCD can be fragmented at two cut sites and yCD activity can be restored by protein complementation when fused to GCN4 zipper (Figure 10). Two cut sites (cut site 3 and 4), which generated yCD PCA activity, were located in a loop region between α2-helix and β3-strand (Figure 7). Cut site 2 and cut site 5, also found in the same loop region as cut site 3 and 4, were observed to have no yCD PCA activity (Figure 10). Perhaps, residues 76-77-78 (of cut sites 3 and 4) are further extended from the enzyme's active site whereas residues 73-74 (of cut site 2) and residues 80-81 (of cut site 5) are packed closer to the enzyme's active site. It is also interesting to note that no yCD PCA activity was found for cut site 6 and cut site 7. Fragment 2 of cut site 7 corresponds to helix 5 and helix 6 of yCD and has been proposed to play an important role in stabilizing the substrate for catalysis (Ireton, Black et al. 2003). This could explain why Fragment 1 (amino acid 1 to 132) of cut site 7 alone is not sufficient to restore yCD activity. In brief, yCD PCA fragmented at cut site 4 gives a better activity than yCD PCA fragmented at cut site 3. When 5000 cells were assayed for their relative

percentage growth inhibition (Figure 10), cells carrying yCD PCA at cut site 4 were approximately 50% inhibited of whereas cells carrying yCD PCA cut site 3 were approximately 30% inhibited. For this reason, further characterization of yCD PCA was performed using cut site 4.

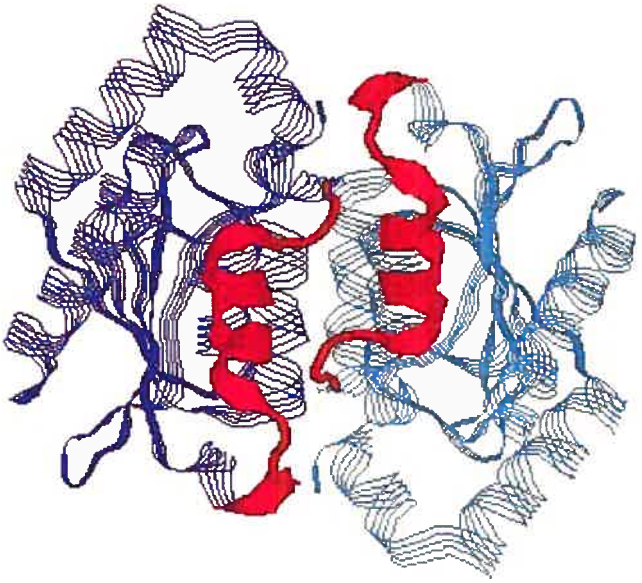
4.3 Overlapping Fragment yCD PCA

Many previous studies have reported that overlapping peptide sequences can contribute to the folding of complementary enzyme fragments and to increase enzyme activity (Ostermeier, Nixon et al. 1999; Wehrman, Kleaveland et al. 2002). In addition, some studies also indicate that an increase of enzymatic activity can be observed from library-generated fragment complementation by removal of some residues from the full length sequence (Ostermeier, Nixon et al. 1999). We fragmented yCD at seven different cut sites and fused each fragment to GCN4 zipper. We then transformed the constructs containing the fragment 1 in the *MAT***a** haploid strain and constructs containing the fragment 2 in the *MAT*α strain. We subsequently combined each fragment 1 with each fragment 2 by mating the corresponding *MAT***a** and *MAT*α clones in order to determine if a certain fragment 1 and fragment 2 combination could resulted in increased yCD PCA activity (Figure 11). From this experiment, we found that GCN4 zipper fused to fragment 1 of cut site 2, 3, and 4 paired with GCN4 zipper fused to fragment 2 of cut site 1, gave better activity than the previously determined GCN4 zipper fused to fragment 1 and fragment 2 at cut site 4 (Figure 11 B). A common element, found in these three new yCD PCA combinations, is a sequence containing two overlapping α2 helices (Figure 7). The combination of Zip-[F1]yCD cut4 and Zip-[F2]yCD cut1, was further characterized and found to have an increased yCD PCA activity determined by both survival and death assay in comparison to Zip-[F1]yCD cut4 and Zip-[F2]yCD cut4.

yCD was purified by gel filtration (Yergatian, Lee et al. 1977) and crystallized (Ireton, Black et al. 2003; Ko, Lin et al. 2003) as a homodimer. It is possible that the

Figure 17. Structure of yCD. Two yCD monomers (one in blue and one in cyan) form a homodimer associated in a head-to-tail manner. The amino terminal (N-terminal) is at the opposite side of the dimer interface. The carboxyl terminal (C-terminal) faces the dimerizing interface. Residues 57 to 77 from the overlapping α2 helices yCD PCA are colored in red and are part of the dimer interface.

C-terminus



N-terminus

N-terminus

C-terminus

dimeric configuration of this enzyme represents the active form of the enzyme. If this hypothesis is true, then having a repetition of two α2 helices could mimic and stabilize the dimeric form of yCD. Analysis of the yCD crystal structure shows that these overlapping α2 helices are involved in the dimer interface (Figure 17). However, these two hypotheses need to be tested. At the moment, we do not know how this overlapping helix can contribute to the increase in yCD PCA activity. We could introduce mutations that disrupt the dimerization interface of yCD, converting it to a monomeric enzyme, and assay for yCD activity. This experiment will reveal if the dimerization of yCD is required in order for the enzyme to deaminate its substrate. Mutations in the overlapping α2 helices can also be done in order to prevent each α2 helix from interacting with each other. The mutant form of yCD PCA containing the non-overlapping α2 helices can be tested for yCD PCA activity using the death assay.

4.4 **Enhanced yCD PCA activity in Yeast**

We have established a solid selection condition with yCD PCA at cut site 4 and found that fragmentation at this site generated significantly decrease in colony numbers after 18 hrs pre-incubation with 100 μg/ml of 5-FC and plating on solid selection medium (Figure 11). Using these conditions for the death assay on solid medium, we compared yCD PCA at cut site 4 and yCD PCA with overlapping α2 helices (Zip-[F1]yCD cut4 and Zip-[F2]yCD cut1) and found that yCD PCA with overlapping α2 helices generated a better activity (Figure 13). This increase in yCD PCA activity was confirmed by a survival assay on solid medium (Figure 14).

We tested the overlapping α2 helices yCD PCA using Ras and RBD as interacting protein partners. We found that the activity of the overlapping α2 helices yCD PCA is dependent on the interaction of Ras and RBD. The combination of Ras-[F1]yCD and Zip-[F2]yCD PCA (Figure 13 h) showed no yCD PCA activity. Cells were resistant to 5-FC similarly to the mock control (Figure 13 f). This could imply that the overlapping yCD PCA signal was observed due to the specific interaction induced by the interaction Ras and RBD rather than yCD fragments complementing by themselves. This characteristic of yCD PCA allows it to be considered for the development of an inhibitor screening system. However, some colonies were observed on the plate containing 1000 μg/ml of 5-FC

(Figure 13 g) for Ras-[F1]yCD and RBD-[F2]yCD whereas no colonies were observed for Zip-[F1]yCD and Zip-[F2]yCD PCA (Figure 13 c) under the same selection condition. This was probably due to the fact that the recombinant proteins Ras-[F1]yCD cut4 and RBD-[F2]yCD cut1 were not as stable as Zip-[F1]yCD cut4 and Zip-[F2]yCD cut1 recombinant proteins when expressed in budding yeast. Some of the Ras-[F1]yCD recombinant protein was observed to be degraded by Western Blot analysis using antibodies [c-H-ras (Ab-1) purchased from Calbiochem] specific against Ras.

4.5 **yCD PCA in Mammalian Cell Lines**

yCD PCA can be applied to many mammalian cell systems as a positive or negative selection method since there is no endogenous cytosine deaminase enzyme in higher eukaryotes. For the positive selection assay, PALA, an inhibitor of aspartate carbamyl transferase (CADases), must be added in order to inhibit the *de novo* pyrimidine synthesis and allow the yCD to become the essential regulatory enzyme for cell survival (Wei and Huber 1996). In the negative selection assay, the introduction of a trans-gene system with yCD PCA that could specifically deaminate 5-FC to 5-FU, serves as a strategy to specifically kill a population of cells. Cells carrying empty vectors have a low level of sensitivity to high concentration of 5-FC.

In this work, our goal was also to show an application of yCD PCA in mammalian cell systems. We took advantage of the death assay in order to increase specificity for killing cancer cells. Many studies used the promoter sequences of genes that are over-expressed in cancer cells to drive the expression of a toxic gene such as yCD or HSV1-TK. Here, we proposed to increase the specificity for targeting cancer cells by using yCD PCA with fragments expressed under the control of different over-expressed genes. The use of two tumor marker promoters and yCD PCA would allow a better specificity in killing tumor cells and decrease the elimination of normal cells which sometimes express one of the tumor marker genes.

We found that the yCD PCA was most effective with individual fragments contained the overlapping α2-helices. When two different tissue specific promoters drove

expression of the two complementary fragments, we could inhibit the growth of HEK 293 cells by approximately 30.5% when treated with 1000 μg/ml 5-FC. Cells carrying WinA-[F1]yCD cut 4 or WinB-[F2]yCD cut1 alone were less sensitive to 1000 μg/ml 5-FC than cells carrying the yCD PCA. Interestingly, our data also suggest that cells carrying WinA-[F1]yCD cut4 alone, under the control of the CMV promoter, were less sensitive to 5-FC than cells carrying empty vectors. This effect was observed for cells carrying WinA-[F1]yCD cut4 alone under the control of the CEA promoter as well but at a less pronounced level. Perhaps, high level expression of WinA-[F1]yCD cut4, when driven by the CMV promoter, influences cells to become less sensitive to 5-FC. However, how this occurs is not obvious. This experiment should be repeated in order to confirm that the observation is not an artifact. In addition, we do not know if WinA, [F1]yCD cut4, or the combined WinA-[F1]yCD cut4 fusion protein is responsible for making cells less sensitive to 5-FC. We could narrow down the possibility by transfecting cells with Zip-[F1]yCD cut4 and determining if this effect can still be observed.

We tested for the expression of the recombinant proteins in stably transfected HEK 293 cells by western blot analysis, using a polyclonal antibody against yCD (purchased from Biogenesis). We found that WinA-[F1]yCD cut4, WinB-[F2]yCD cut1, and WinA-yCD were detected when expressed under the control of CMV promoter, but not detected when expressed under the control of CEA or hTERT promoter (data not shown). Since we were unable to detect yCD activity in the latter stably transfected cells (Figure 16), it is possible that WinA-[F1]yCD cut4, WinB-[F2]yCD cut1, and WinA-yCD were expressed, but at a low level, beyond the capacity of detection of this antibody. Interestingly, the expression of yCD under CEA promoter has previously been reported using another antibody against yCD (Nyati, Sreekumar et al. 2002). Perhaps the antibody used by Nyati et al. has a higher affinity for yCD. We would like to try this antibody on our stably transfected cells in order to confirm that they expressed the recombinant protein (s). However, it is also possible that the fusion proteins, WinA-[F1]yCD cut4, WinB-[F2]yCD cut1, and WinA-yCD, are less stable than the wild type yCD and therefore, harder to detect using antibody against yCD.

In general, our findings suggest the possibility of using yCD PCA for targeting a population of mammalian cells for death. However, at this stage, the yCD PCA is not efficient for inhibiting cell growth since a high concentration of 5-FC must be used in order to obtain an increased of 10 to 15 % growth inhibition in comparison to the mock cells. We attempted the death assay for yCD PCA in mammalian cells using 100 μg/ml 5-FC. Unfortunately, no significant growth inhibition was observed (data not shown).

4.6 Conclusions

We have developed the first Binary Positive and Negative PCA using yCD, which can be used to increase the specificity of a survival selection assay or a cell growth inhibition assay. In addition, the Binary Positive and Negative PCA can be used as a screening tool for interacting protein partners. We chose to establish the assay in yeast and observed the highest yCD PCA activity in the survival and death selection assay using yCD fragments with 20 overlapping amino acid residues located on the second alpha-helix of the enzyme. We applied yCD PCA under CMV and tissue specific promoters in mammalian cells and found that cell growth inhibition was slightly inhibited using high concentration of 5-FC.

4.7 Perspectives

At present, we are in the process of generating and testing yCD PCA activity in CaCo-2 stable cell lines. Based on results obtained from our promoter re-characterization assay (Figure 15), the promoter activity of CEA and hTERT promoters were similar for HEK 293 and CaCo-2 cells. Therefore we suspect that yCD PCA activity in CaCo-2 stable cell lines might be similar to that observed in the HEK 293 stable cell lines based on expression levels.

For future projects, further optimization of yCD PCA to increase the 5-FC deamination activity is required in order to apply this PCA in mammalian cells. Although some yCD PCA activity was observed in mammalian cells when high concentration of 5-FC was added, the activity of yCD PCA was weaker than the full-length enzyme. A

library of yCD fragment 1 and fragment 2 can be generated by using DNA shuffling or error-prone PCR strategy. The selection of a functional yCD PCA from the library-generated fragments should be easy to achieve since the survival selection assay of yCD PCA can be used. The new yCD PCAs can then be screened for an increase in 5-FC deamination activity using the death assay.

Once the engineered yCD PCA with an improved 5-FC deamination activity is well characterized, it can be used for applications in mammalian cells. The engineered yCD PCA can be used in combination with tissue specific promoters to specifically kill a population of cells. The engineered yCD PCA can also be used to select for a specific clonal population of cells with the survival assay.

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